Genetics of Male Infertility

A Case-Based Guide for Clinicians

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Editors
Mohamed Arafa
Department of Urology
Hamad Medical Corporation
Doha, Qatar

Haitham Elbardisi
Department of Urology
Hamad Medical Corporation
Doha, Qatar

Ahmad Majzoub
Department of Urology
Hamad Medical Corporation
Doha, Qatar

Ashok Agarwal
Cleveland Clinic
Cleveland, OH
USA

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Foreword

The treatment of male infertility is progressively changing. One of the areas where new knowledge could lead to impressive new therapeutic opportunities is in the area of genetics of male fertility. This book *Genetics of Male Infertility* is presented as a case-based discussion of conditions that may affect male fertility. The topics covered in the book are broad-ranging and relevant for the understanding of all conditions that are important for management of the infertile male. Each chapter has a broad overview of the subject matter, complete with literature search. Each chapter provides a structured topic discussion that is often emphasized with a case discussion, where the condition being overviewed is tied to a specific clinical scenario to enhance the clinical relevance of the chapter.

Some topics are of general information that are of importance for a complete understanding of male reproductive function. For example, Chaps. 1, 4, 7, 17, 18, and 19 are critical chapters that provide key foundations of genetic factors that are involved in male reproductive development or function. They emphasize the potential to develop better diagnostic genetic testing that will allow optimal understanding of male infertility.

Other chapters, such as Chaps. 2, 8, 10, 16, and 22, provide discussion that revolve around areas that are important for the understanding of the genetics of male infertility but are not yet applicable for the treatment of infertile men. Each of these areas provide a critical foundation for the development of new interventions in male reproduction. Without a critical understanding of the molecular basis of spermatogenesis, for example, we have little chance of providing a molecular or therapeutic intervention for men with maturation arrest where spermatozoa cannot be retrieved from the ejaculate or testicular tissue. The genetic intervention that could be possible with CRISPR technologies requires a greater understanding of the underlying genetic etiologies of so many men with male infertility, especially for men with the most severe forms of male infertility such as non-obstructive azoospermia. Even stem cell-based technologies may not be applicable to infertile men if we don't have a better understanding of the genetic causes of male reproductive dysfunction.

Topical clinically relevant conditions important for clinicians and scientists interested in male fertility issues include Chaps. 3, 5, 6, 9, 11, 12, 13, 14, 15, 20, and 21.

These chapters are critical for assessing clinical management of these genetic conditions and their effective management.

Each of these chapters provide a background that is important for the male reproductive expert. Taken together, they provide an excellent foundation of knowledge in the genetics of male reproduction.

Peter N. Schlegel, MD
Weill Cornell Medicine
New York, NY, USA

Preface

Infertility is a major public health concern affecting up to 190 million couples worldwide. It can result from a wide array of etiologies that are related to male and/or female factors, with the male factor contributing in up to 50% of cases. The extensive research seen in the field of reproductive medicine in the last two decades has exposed a great deal of the pathophysiologic mechanisms resulting in infertility; however, despite all efforts, in as many as 30% of infertile couples, the etiology remains unknown. It is reasonable to consider that many of these cases of idiopathic infertility may have a clear genetic basis.

Genetic disorders in males can lead to impaired spermatogenesis, defective sperm function, and defects in delivery of sperm. From a clinician's point of view, genetic causes and treatments of male infertility are usually hard to understand, interpret, and implement in the clinical setting. Therefore, the main aim of this book is to bridge all of these difficulties and present genetic abnormalities in male infertility and their treatment as an easy applicable clinical management strategy. Furthermore, an understanding of the genetic basis of male infertility allows for the appropriate counseling of patients about treatment options and risks to their potential offspring.

This textbook is unique as it provides a thorough review of the genetic causes of male infertility. Forty-three experts from 11 different countries have contributed to make this book an important reference guide in the field of reproductive medicine. The book is divided into four sections. Part I explores the genetic foundation of male reproduction, while Part II discusses the various implications of genetic abnormalities on sperm quality and male infertility. Part III presents various genetic etiologies for male infertility in clinical case scenarios highlighting their presentations, as well as the diagnostic and therapeutic modalities that can be offered for each case. Finally, Part IV presents an overview of future directions in managing the genetic causes of male infertility.

We are confident that our book will be a useful guide for clinicians, geneticists, scientists, embryologists, and other healthcare workers engaged in the care of infertile couples. In addition, it will be a valuable resource for students and researchers wishing to learn more about this subject. We are highly thankful to the large number

of experts who worked hard to contribute the latest, well-written, and well-researched manuscripts; without their active support, this book would not be possible. We wish to express our deep gratitude to the organizational and management skills of Elizabeth Orthmann, Springer Development Editor, and to Kristopher Spring, Senior Editor at Springer, for his supervision and overall support of this project. This book is dedicated to our parents, families, mentors, and patients.

Doha, Qatar — Mohamed Arafa
Doha, Qatar — Haitham Elbardisi
Doha, Qatar — Ahmad Majzoub
Cleveland, OH, USA — Ashok Agarwal

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Author Biographies

Mohamed Arafa graduated from Cairo University Medical School in 1996. After he finished his Internship at Kasr Al Aini Hospital, Cairo University, he joined first as a resident and then as a medical staff in the Andrology Department. He is now working as an Associate Professor of Andrology and STDs, Cairo University. From 2004 till 2011, he worked as the Head of Andrology and STDs in Dr Fakhry and Al Rajihi Hospital in Al Khobar. Since 2011, he is working as Consultant Urology/Male Infertility at Hamad General Hospital, Qatar. He is appointed as Adjunct Assistant Professor of Urology, Weill Cornell Medicine – Qatar. He is recently appointed as Assistant Scientist at the American Center for Reproductive Medicine, Cleveland Clinic, Cleveland, Ohio, USA.

During his profession in andrology, Dr Arafa has shown great interest in male infertility diagnosis and treatment. He has respectable experience in andrology laboratory procedures, including routine and advanced semen analysis and sperm cryopreservation. His surgical skills extend to cover all andrology surgeries, especially microsurgical procedures (testicular biopsy, varicocelectomy, vasoepididymostomy, and vasovasostomy).

Dr Arafa is a reviewer for a number of international journals dealing with andrology. He was an Ordinary Director in the Middle East Society for

Sexual Medicine (MESSM) Board of Directors and the Chief Editor of *MESSM Newsletter*. He is a fellow of the Multidisciplinary Joint Committee on Sexual Medicine in 2012.

The research activities of Dr Arafa include more than 100 published articles in international peer-reviewed journals and many book chapters with h-index of 18 on Google Scholar and with 1068 citations. His research covers all domains of andrology, mainly genetics and proteomics of male infertility, oxidative stress in semen, sexual dysfunctions, and late-onset hypogonadism.

Haitham Elbardisi graduated from Medical School in Cairo University, Egypt, in 1996. He began his career in urology at Hamad Medical Corporation (HMC) when he joined the residency program in 2002. He has been an Arab Board Certified Urologist since 2008. He also showed interest in andrology, especially male infertility. He finished the Andrology Fellowship Program at HMC in 2012. In 2012, he also became a fellow of the European Multidisciplinary Joint Committee of Sexual Medicine.

Since 2009, Dr Haitham has specialized in male infertility at Hamad General Hospital (HGH). HGH is the main governmental hospital in Qatar where the only specialized male infertility section and the main IVF Centre in Doha are located. With his male infertility team, Dr Haitham has succeeded in mastering all male infertility domains, including advanced diagnostic techniques and medical and surgical treatment. Dr Haitham is an expert in microsurgical male infertility procedures including varicocelectomy, reconstructive surgery, vasectomy reversal, vasoepididymostomy, and testicular biopsy.

Dr Haitham is involved in the academic program in urology where he holds the position of Associate Program Director of the Urology Residency Program since 2011. He has more than 50 publications in peer-reviewed journals and many chapters in all domains of andrology with h-index of 11 on Google Scholar and 329 citations.

Ahmad Majzoub is a specialist in the fields of andrology and male infertility. He is currently working as a Consultant at the Department of Urology and Program Director of Andrology and Male Infertility Fellowship at Hamad Medical Corporation, Doha, Qatar. He is also affiliated to Weill Cornell Medicine – Qatar, holding a position of Assistant Professor. He obtained his MBChB from Beirut Arab University in 2005 and proceeded to complete his residency training in urology in Qatar to become an Arab Board Certified Urologist. Dr Majzoub underwent a specialized two-year training in Andrology and Male Infertility at the Hamad Medical Corporation and did a second Clinical and Research Fellowship in Andrology at the world-famous Glickman Urological and Kidney Institute and the American Center for Reproductive Medicine at Cleveland Clinic Foundation, Cleveland, USA. He is also a fellow of the European Committee of Sexual Medicine.

Dr Majzoub provides services in general urology, andrology, and male infertility offering endoscopic, microsurgical, reconstructive, and prosthetic surgeries for his patients. His interests extend towards the management of chronic pelvic pain syndrome in men and has opened the first dedicated pelvic pain clinic in the region.

Dr Majzoub has done extensive work in the field of medical research and has been very active with over 120 research publications in peer-reviewed journals and several book chapters mainly focusing on andrology and men's health. His Hirsch index (h-index) as of July 2019 is 12 on Google Scholar with a citation count of 795. According to ResearchGate, Ahmad has an RG score of 33.59 on 153 publications and 790 citations.

He is a reviewer at several high-impact medical journals and is an active member of the American Urological Association, the American Association for Reproductive Medicine, the Society for the Study of Male Reproduction, the European and International Societies of Sexual Medicine, and the Arab Association of Urology. He holds editorial positions at a number of scientific journals and has had several speaker participations at national and

international conferences. Ahmad has served as an editor of two special issues in scientific journals on topics related to male infertility and is also a co-editor of five textbooks focusing on sperm cryopreservation, varicocele, sperm retrieval and preparation, sperm functional studies, and genetics of male infertility. He recently founded the Men's Virtual Clinic, an online platform dedicated for male patient's education regarding their sexual and reproductive health.

Ashok Agarwal, PhD, HCLD (ABB), ELD (ACE), EMB (EMBCOL), is the Head of Andrology Center and Director of Research at the American Center for Reproductive Medicine since 1993. He holds these positions at The Cleveland Clinic Foundation, where he is Professor of Surgery (Urology) at the Cleveland Clinic Lerner College of Medicine of Case Western Reserve University. He was trained in Male Infertility and Andrology at the Brigham and Women's Hospital and at Harvard Medical School and later worked as Assistant Professor of Urology at Harvard from 1988 to 1992. He has over 26 years of experience in directing busy male infertility diagnostic facilities and fertility preservation services. He is very well published with over 730 scientific papers and reviews in peer-reviewed scientific journals and is ranked in Scopus as the #1 author in the world in the fields of Male Infertility/Andrology and Human-Assisted Reproduction, based on the number of peer-reviewed publications, citation scores (Scopus, 32,001; Google Scholar, 67,039), and h-index (Scopus, 96; Google Scholar, 125). He is currently an Editor of 39 medical textbooks/manuals related to male infertility, ART, fertility preservation, DNA damage, and antioxidants and active in basic and clinical research. His laboratory has trained over 1,000 scientists, clinicians, and graduate and undergraduate students from the United States and more than 55 countries. His current research interests include proteomics of male infertility and the molecular markers of oxidative stress and DNA integrity in the pathophysiology of male reproduction.

Contributors

Ibrahim A. Abdel-Hamid, MD Department of Andrology, Mansoura Faculty of Medicine, Mansoura University Hospital, Mansoura, Egypt

Ashok Agarwal, PhD American Center for Reproductive Medicine, Cleveland Clinic, Cleveland, OH, USA

Mohamed Arafa, MD Department of Urology, Hamad Medical Corporation, Doha, Qatar

Kenneth I. Aston, PhD Department of Surgery, University of Utah School of Medicine, Salt Lake City, UT, USA

Elisabetta Baldi, PhD Experimental and Clinical Medicine, University of Florence, Florence, Italy

Joshua Bitran, BS Department of Urology, University of Miami, Miami, FL, USA

Douglas T. Carrell, PhD Departments of Surgery (Andrology) and Human Genetics, University of Utah School of Medicine, Salt Lake City, UT, USA

Julie W. Cheng, MD, MA Department of Urology, Loma Linda University Health, Loma Linda, CA, USA

Ronald G. Crystal, MD Department of Genetic Medicine, Weill Cornell Medical College, New York, NY, USA

Simon Dadoun, BS Department of Urology, University of Miami, Miami, FL, USA

Igor Faria Dutra, MD Department of Reproductive Medicine, ORIGEN-Center for Reproductive Medicine, Rio de Janiero, Brazil

Department of Surgery, Division of Urology, Antonio Pedro University Hospital, Fluminense Federal University, Rio de Janiero, Brazil

Haitham Elbardisi, MD Department of Urology, Hamad Medical Corporation, Doha, Qatar

Moustafa A. Elsaied, MD Department of Andrology, Mansoura Faculty of Medicine, Mansoura University Hospital, Mansoura, Egypt

Ezzat S. Elsobky, MD Genetics Unit, Pediatrics Department, Ain Shams University, Medical Genetics Center, Cairo, Egypt

Khalid A. Fakhro, PhD Department of Genetic Medicine, Weill Cornell Medicine – Qatar, Doha, Qatar

Human Genetics Department, Sidra Medicine, Doha, Qatar

David Fisher, PhD Department of Medical Bioscience, University of the Western Cape, Bellville, South Africa

Rahul Krishnaji Gajbhiye, MBBS, PhD Department of Clinical Research, ICMR-National Institute for Research in Reproductive Health, Mumbai, Maharashtra, India

Jingtao Guo, PhD Huntsman Cancer Institute and Andrology Lab, Department of Oncological Sciences and Surgery, University of Utah School of Medicine, Salt Lake City, UT, USA

Ralf Henkel, PhD Department of Medical Bioscience, University of the Western Cape, Bellville, South Africa

Emma Rae James, BS Department of Surgery, University of Utah, Salt Lake City, UT, USA

Timothy G. Jenkins, PhD Department of Surgery, University of Utah, Salt Lake City, UT, USA

Meghali Joshi, Mtech Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh, India

Kareim Khalafalla, MD, MBBCh Department of Urology, Hamad General Hospital, Doha, Qatar

Shagufta Khan, PhD Department of Clinical Research, ICMR-National Institute for Research in Reproductive Health, Mumbai, Maharashtra, India

Edmund Y. Ko, MD Department of Urology, Loma Linda University Health, Loma Linda, CA, USA

Joseph T. Mahon, MD Genitourinary Reconstruction & Men's Pelvic Health, Department of Urology, Loyola University Medical Center, Maywood, IL, USA

Ahmad Majzoub, MD Department of Urology, Hamad Medical Corporation, Doha, Qatar

Vineet Malhotra, MD Department of Urology and Andrology, Diyos Hospital, New Delhi, Delhi, India

Sara Marchiani, PhD Andrologia, Endocrinologia femminile e Incongruenza di genere- Dipartimento Materno Infantile, Azienda Ospedaliero Universitaria Careggi, Florence, Italy

Marlon P. Martinez, RMT, MD Philippine Urological Association, Department of Surgery, Section of Urology, University of Santo Tomas Hospital, Sampaloc, Manila, Philippines

Deepak Modi, PhD Molecular and Cellular Biology Laboratory, National Institute for Research in Reproductive Health – Indian Council of Medical Research, Mumbai, Maharashtra, India

Monica Muratori, PhD Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy

Manesh Kumar Panner Selvam, PhD American Center for Reproductive Medicine, Cleveland Clinic, Cleveland, OH, USA

Robert A. Petrossian, MD Department of Urology, Southern Illinois University School of Medicine, Springfield, IL, USA

Ranjith Ramasamy, MD Department of Urology, University of Miami, Miami, FL, USA

Amal Robay, PhD Department of Genetic Medicine, Weill Cornell Medicine – Qatar, Doha, Qatar

Juan L. Rodriguez-Flores, PhD Department of Genetic Medicine, Weill Cornell Medical College, New York, NY, USA

Matheus Roque, MD, PhD Department of Reproductive Medicine, Mater Prime – Reproductive Medicine, São Paulo, Brazil

Peter N. Schlegel, MD Department of Urology, New York Presbyterian/Weill Cornell, New York, NY, USA

Pallav Sengupta, PhD Department of Physiology, Faculty of Medicine, Bioscience and Nursing, MAHSA University, Jenjarom, Selangor, Malaysia

Rupin Shah, MS, MCh Department of Andrology, Lilavati Hospital and Research Centre, Mumbai, Maharashtra, India

Neha Singh, MSc Molecular and Cellular Biology Laboratory, National Institute for Research in Reproductive Health – Indian Council of Medical Research, Mumbai, Maharashtra, India

Rajender Singh, PhD Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh, India

Nicholas N. Tadros, MD Department of Urology, Southern Illinois University School of Medicine, Springfield, IL, USA

Lara Tamburrino, PhD Experimental and Clinical Medicine, University of Florence, Florence, Italy

Lorena Rodrigo Vivó, PhD PGD Molecular Cytogenetics, Igenomix, Valencia, Spain

Part I
The Genetic Basis of Reproduction

Chapter 1
The Molecular Genetics of Testis Determination

Neha Singh and Deepak Modi

Abbreviations

ALCs	Adult Leydig cells
AMH	Anti-Mullerian hormone
ATRX	Alpha thalassemia/mental retardation syndrome
BPES	Blepharophimosis/ptosis/epicanthus inversus syndrome
CAH	Congenital adrenal hypoplasia
CBX2	Chromobox homolog 2
CGD	Complete gonadal dysgenesis
CYP26B1	Cytochrome P450, family 26, subfamily b, polypeptide 1
DAX1	DSS AHC critical region on the chromosome X
DAZL	Deleted in azoospermia-like
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4
DHH	Desert hedgehog
DMRT1	dsx- and mab3-related transcription factor 1
DSD	Disorders of sex development
E	Embryonic day
EMX2	Empty spiracles homeobox 2
FGFR2	FGF receptor 2
FLCs	Fetal Leydig cells
FOG2	Friend of GATA-2
FOXL2	Forkhead box L2
GADD45G	Growth arrest and DNA damage-inducible 45 G
GATA4	GATA binding protein 4

N. Singh · D. Modi (✉)
Molecular and Cellular Biology Laboratory, National Institute for Research in Reproductive Health – Indian Council of Medical Research, Mumbai, Maharashtra, India
e-mail: modid@nirrh.res.in

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GD	Gonadal dysgenesis
HMG	High-mobility group
LHX9	LIM homeobox 9
LOF	Loss of function
NR0B1	Nuclear receptor subfamily 0, group B, gene 1
NR5A1/SF1	Nuclear receptor subfamily 5, group A, gene 1/steroidogenic factor 1
PGCs	Primordial germ cells
PGD	Partial gonadal dysgenesis
PMCS	Peritubular myoid cells
PTCH1	Patched receptor 1
RSPO1	R-spondin family 1
SIX1/4	SIX homeobox 1/4
SOX	Sry-related HMG box
SOX10	Sry-related HMG box 10
SOX9	Sry-related HMG box 9
SRY	Sex-determining region on Y chromosome
WES	Whole exome sequencing
WNT4	Wingless-type MMTV integration site family, member 4
WT1	Wilms' tumor 1

Key Points

- Sex determination is a decision by which fate of bipotential gonad is decided either into ovary or testis.
- *SRY* gene, on the Y chromosome, is the master regulator of testis determination.
- SRY first specify Sertoli cells, and subsequently Leydig cells, endothelial cells, peritubular myoid cells, and interstitium differentiate, following the decision.
- Leydig cells produce testosterone, leading to differentiation of vas deferens and epididymis, and Sertoli cells produce AMH for regression of Mullerian ducts.
- Testosterone also aids in differentiation of the scrotum and penis leading to masculinization.

Background

In adult humans, the two sexes are physically different; however, this is not true at the start of life. At 5 weeks of development in humans [1] and almost mid-gestation in mouse (E9.5 of 21-day gestation), the embryos develop both the male and female structures [2]. Next, to the developing renal system, two bulges of the gonadal ridges emerge along with two pairs of ducts, viz., the Mullerian ducts that form the

uterus and fallopian tubes and the Wolffian duct which is the analgen of the epididymis, vas deferens, and seminal vesicles of the male reproductive system. At 6 weeks of development in humans and E10.5 in the mouse, the gonad switches on the developmental pathways that result in the formation of either an ovary or a testis. If a testis develops, it secretes testosterone for the development of the male ducts and also directs the development of the external genitalia to masculinize into a scrotum and a penis. The testes also secrete the anti-Mullerian hormone (AMH) for the regression of the Mullerian derivatives. If the gonad differentiates into an ovary, the lack of testosterone causes the Wolffian ducts to regress, and the absence of AMH allows the retention of the Mullerian derivatives that later differentiate into the female reproductive system. This entire process of development of the gonad from a bipotential state is termed as sex determination, and the maturation of the gonad and development of the respective duct system is termed as sex differentiation. Changes in any of these processes can have dramatic effects on the development of an individual's sex (phenotype), resulting in disorders of sex development (DSD).

Assembly of the Bipotential Gonads

During embryonic development, mammalian gonad develops from thickening of the coelomic epithelium. The gonad consists of mesonephros and genital ridge which comprises of somatic cells from proliferating epithelia and migrating cells from adjacent mesonephros. These cells contribute to the Sertoli or the granulosa cells and steroid-producing cells of the adult gonads. Interestingly, this gonad does not contain the germ cells which are the future spermatogonia or oogonia. Germ cells are extraembryonic in origin that migrate from the base of allantois through hindgut and colonize the gonad. It is important to remember that irrespective of the chromosomal sex of the embryo XX or XY, the gonad that is developed is always bipotential, i.e., it has the ability to differentiate either into a testis or an ovary.

Several genes have been identified that are required for the development of the bipotential gonads. These include *Lhx9*, *Wt1*, *Gata4*, *Sf1* or *Nr5a1*, *Cbx2 (M33)*, *Emx2*, *Six1*, *Six4*, and genes encoding insulin receptors (*Igf1r/Irr/Ir*). Loss of these genes in mouse causes failure to develop the bipotential gonad; however, these mice often show other associated phenotypes, mainly renal agenesis, as most of these genes are also required for formation of renal primordium. Table 1.1 summarizes the mouse genes whose loss results in failure of gonad formation leading to gonadal dysgenesis and sterility. Among these genes, mutations in *WT1* in humans cause WAGR syndrome (Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation), Denys-Drash syndrome (including gonadal abnormalities and renal failure), and Frasier syndrome (46,XY gonadal dysgenesis together with glomerulopathy) that have been associated with DSD [3]. Whether the other genes required for mouse bipotential gonad development are also required in humans is not known.

Table 1.1 Genes involved in testis development

Stage	Genes required
Formation of bipotential gonad	*Lhx9*, *Wt1*, *Gata4*, *SF1*, *Cbx2*, *Emx2*, *Igrf1/Igr/Irr*
Sertoli cell specification	*Sry*, *Gadd45g*, *Fog2*, *Gata4*, *Six1*, *Six4*
Differentiation and tubulogenesis	*Sox9*, *Sox10*, *Fgf9*, *Pdgfr*, *Pdgs*, *Dmrt1*, *Amh*
Vascularization	*VEGF*, *FGF*, *Robo4*, *Flk1*, *Jag1*, *Pdgfr*
Leydig cell specification and function	*Pdgfr alpha*, *Dhh/patched*, *Arx*, *androgen receptors*
Germ cell sex determination	*Ckit*, *SSea1*, *Vasa*, *DazL*, *Nanos2*, *Cyp26b1*

This could be perhaps due to the fact that these genes are required in many other organ system development and mutations in them may be embryonically lethal.

Sex Determination

Once the bipotential gonads are formed, they have to commit to either of the two sexes. The process of the commitment of bipotential gonad to the testicular or ovarian fate is termed as sex determination. In humans and most mammals, the Y chromosome is a dominant sex-determining factor due to the presence of *SRY* (sex-determining region on Y) gene. *SRY* belongs to a high-mobility group (HMG) box of transcription factor that controls target gene expression by binding to and bending DNA. *SRY* was first identified as the testis-determining gene in humans by screening of phenotypic males who had a 46,XX karyotype. A small 35-kilobase (Kb) region of the human Y chromosome was identified that translocated to the X chromosome in these individuals [4]. Analysis of this sequence identified a 1 Kb intron less gene that was termed as *SRY*. The proof that *SRY* is an authentic sex-determining gene came from studies in the mouse where XX embryos, when injected with the *SRY* gene, developed as males and had testis in place of the ovaries [5]. That *SRY* is an authentic testis-determining gene is further evident from the studies in patients with Swyer syndrome where nearly 15% of the cases have either deletion or inactivating mutations in the *SRY* gene. Presenting as XY gonadal dysgenesis, Swyer syndrome is a type of hypogonadism where the individual karyotype is 46,XY but is externally female with streak gonads. Swyer syndrome is also associated with mutations in other genes involved in *SRY* activation (see below) and those involved in Sertoli cell development. These include *ARX*, *ATRX*, *CBX2*, *DHH*, *DMRT1*, *GATA4*, *MAMLD1, MAP3K1*, *NR0B1*, *NR5A1*, *SOX9*, *WT1*, *WWOX*, *SRY*, and *WNT4* genes [6].

While we recognize the gonadal sex of an individual by the gamete it produces (sperm for testis and oogonia for the ovary), it is important to remember that sex determination in all species is initially driven by the somatic cells and the rest of the cell types just follow. In all the mammalian species studied to date, *SRY* is expressed by the pre-Sertoli cells. In mouse, *Sry* expression is initiated on E10.5, and the early expression of *SRY* in humans is reported around 6.5 to 7 weeks of gestation [7]. In the mouse, *Sry* acts within a tight developmental window (E10.5 to E12.5) for testis

determination, and any delay can result in male to female gonadal sex reversal or ovotestis development. This brief period of expression is consistent with the role of *Sry* in orchestrating the initial stages of testis determination, but not subsequent differentiation as its expression is extinguished by E12.5. In contrast, human *SRY* is continuously expressed in the Sertoli cells well after sex determination and also in adulthood [7] indicating some species-specific functions of *SRY*.

A number of genes are required for the normal expression of *Sry*. These include *Gata4/Fog2*, *M33 (Cbx2)*, *Six1/4*, *Map3k4*, *Jmjd1a*, and *Gadd45g*. Based on extensive biochemical and genetic studies, three independent modules of genes have been proposed [8] to activate *SRY* in the bipotential gonad (Fig. 1.1). The first module contains the insulin receptors, *Gadd45g*, *Map3k4*, *Six1/4*, and *Fog2* that converge to regulate *Gata4* and activate *Sry* transcription. The second module has *Nr5a1* (also known as *Sf-1*) at its center. This module consists of *Lhx9*, *Cbx2*, *Cited2*, and *Six1/4* (of module 1) which cooperatively activate *Sry*. Finally, the lesser understood module is the *Wt1* module. Loss of *Wt1* results in lowered *Sry* expression resulting in the formation of ovotestis [9], but what activates *Wt1* or regulates its activity is unknown. At this conjuncture, we must remember that our understanding of *Sry* activation comes from studies in mice lacking these genes and their relevance to the activation of human *SRY* is hitherto undetermined. However, it is interesting to note that mutations in *MAP3K4* (a member of module 1), *NR5A1*, *GADD45G,* and *CBX2* lead to 46,XY DSD in humans [6, 10].

Sry activation also involves epigenetic regulation. Correct gene expression while requiring a host of transcription factors also needs the DNA (chromatin) to be made accessible through epigenetic modifications such as histone modifications and demethylation. Sry activation (at least in the mouse) requires the histone demethylase *Jmjd1a* [11]. In human, histone acetyltransferase p300 induces acetylation of

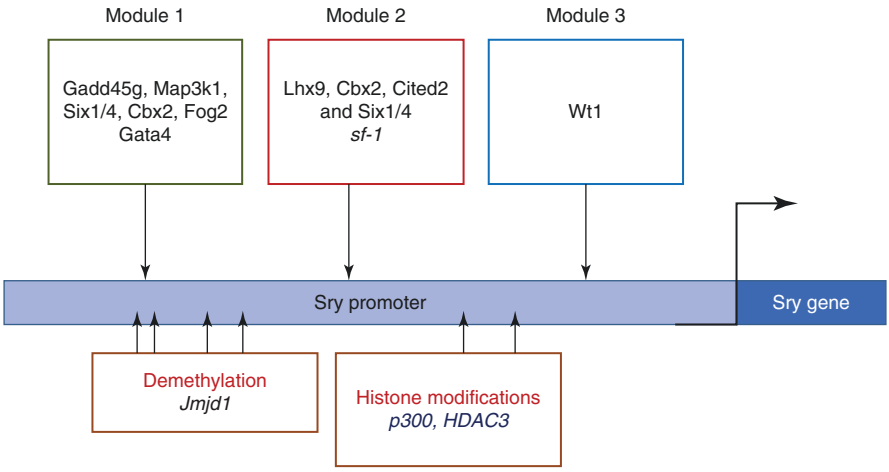

Fig. 1.1 Genes required for activation of *Sry*. The genes that form a network to activate transcription factors *Gata4*, *Sf-1*, and *Wt1* that cooperatively activate Sry in the developing gonads. In addition, Sry transcription requires active demethylation and histone modifications

SRY gene which assists nuclear localization of *SRY*, whereas specific deacetylation by *HDAC3* induces a cytoplasmic delocalization of *SRY* [12], suggesting the importance of histone modifications in regulating *SRY* activity. Beyond histone modifications, DNA demethylation is also associated with *SRY* activation. In mouse gonads, the promoter of *Sry* is hypermethylated just prior to E10.5, it gets hypomethylated in the gonad at E11.5, when *Sry* expression peaks, but remains hypermethylated in tissues that do not express *Sry* [13]. This suggests that demethylation of *Sry* promoter is also required for its activation. However, the demethylase enzyme that is required for this process is unknown.

Sertoli Cell Specification

Once *SRY* is activated, Sertoli cells differentiate from supporting lineage by expressing *SOX9*. SOX9 (*SRY*-box 9) is a HMG box protein necessary and sufficient for testis determination. *Sox9* is expressed in both XY and XX gonads at low levels but is rapidly upregulated in the XY gonad and extinguished in the XX gonad by around E11.5 [14]. Like *SRY*, *SOX9* expression is restricted to pre-Sertoli and Sertoli cells to initiate cord formation. For this process, the Sertoli cells get epithelialized and exhibit apicobasal polarity to organize into seminiferous tubules. Loss of *Sox9* in XY gonads causes XY sex reversal in mice, and forced expression of *Sox9* in XX gonads can cause testis differentiation [15]. Mutations in human *SOX9* causes campomelic dysplasia; a subset of these patients are associated with XY sex reversal [16], underscoring the importance of *SOX9* in human sex determination.

Once SRY activates *SOX9*, it requires fibroblast growth factor 9 (*FGF9*) to maintain its expression [14]. FGF9 is a secreted growth factor, and loss of *Fgf9* in XY mouse embryos results in sex reversal. In these mice, although *Sry* expression is normal and *Sox9* is upregulated, the expression of SOX9 is not maintained in absence of *Fgf9* [14]. Produced by SRY- and SOX9-positive Sertoli cells, FGF9 acts in Sertoli cell differentiation primarily by maintaining SOX9 and opposing pro-ovarian signals [14]. FGF9 also ensures sufficient numbers of Sertoli cells by recruiting cells of the supporting lineage to a Sertoli cell fate and preventing sex reversal. In human females, gain of function of *FGF9* leads to 46,XX testicular DSD [17]. The other genes required for Sertoli cell differentiation are listed in Table 1.1.

Sex Differentiation

There is presently no clear boundary that defines completion of testis determination and initiation of sex differentiation. Anatomically, testis differentiation proceeds by organization of Sertoli cells into seminiferous tubules and production of AMH for regression for Mullerian ducts. At the same time, testis differentiation involves

specification of the Leydig cells for testosterone production and masculinization. Once SRY activates *Sox9*, high SOX9-expressing Sertoli cells aggregate with germ cells known as somatic germ cell mass (SGCM). At this time, germ cells are tightly connected by intercellular bridges by virtue of expressing E-cadherin. This allows the germ cells to form big clusters around which Sertoli cells organize and polarize to form solid testicular cord which is demarcated by basal lamina from mesenchyme. At the same time, migration of endothelial cells lead to vascularization which along with testicular cord partition the mesenchyme. Germ cells seem to act like a scaffold and allow the Sertoli cell alignment and hence tubulogenesis.

Two genes that are at the interface of testis determination and differentiation are *Dmrt1* (doublesex and mab-3-related transcription factor 1) and *Dax1* (*NR0B1*, nuclear receptor subfamily 0, group B, member 1). *DMRT1* is a transcription factor, while *DAX1* is an orphan nuclear hormone receptor. While both *Dmrt1* and *Dax1* are not required for primary sex determination in mice, as XY mice lacking these genes are born with testes [18]. However, in the absence of *Dmrt1*, testicular fate cannot be maintained, and Sertoli cells undergo postnatal reprogramming to granulosa-like cells [19]. Furthermore, forced overexpression of *Dmrt1* in the mouse XX embryonic gonads results in Sertoli cell differentation [20, 21]. Deletions of human chromosome 9p24 (that contains *DMRT*1 gene) and mutation in *DMRT1* gene are associated with 46,XY DSD [6, 10].

Duplications of a region of human Xp21 known as dosage-sensitive sex (DSS) reversal locus which contains *DAX1* are associated with 46,XY gonadal dysgenesis, and excess of DAX1 is thought to inhibit testis determination in these patients [22]. Humans with Xp21 deletions develop adrenal hypoplasia congenita (AHC) where testes develop normally, but individuals have disorganized testis cords and hypogonadotropic hypogonadism [23]. High levels of *DAX1* expression in XY mice can cause gonadal sex reversal [24] by inhibiting the activation of *Sox9* [3].

A hallmark of testis differentiation is production of AMH. Initiated at E12.5 in the mouse and around 7 weeks in humans, AMH production by the Sertoli cells marks its terminal differentiation [25]. That AMH is not required for sex determination or testis differentiation is evident from studies in XY mice that lack *Amh*. In these mice, there is proper Sertoli cell determination and tubule organization, and there are no overt sex reversal phenotypes except that the Mullerian ducts in these XY mice fail to regress [26]. As a result, there is a physical barrier to testicular descent later in gestation which leads to cryptorchidism-induced infertility [27]. In humans, mutations inactivating AMH cause persistent Mullerian duct syndrome (PMDS) in 46,XY, and activating mutations in AMH causes Mullerian agenesis in 46,XX females [6]. Thus, AMH is not required for primary gonadal sex determination or differentiation but is required for proper differentiation of the somatic sex.

Male sex differentiation also requires regression of the Mullerian ducts and proliferation of Wolffian ducts. AMH produced by the Sertoli cells act on the AMH receptors on the Mullerian ducts causing their apoptosis, and the fetal Leydig cells (see below) will produce testosterone which acts on the Wolffian ducts to differentiate into the male duct system and masculinization of external genitalia.

Partitioning of Testis Cords by Mesonephric Cell Migration, Formation of Peritubular Myoid Cell, and Testis Vascularization

Testis morphogenesis requires organization of Sertoli cells into testicular cords. However, these Sertoli cells need to be directed, and multiple cell types from several sources play a central role. The primitive tubular structures are termed as "proto-cords" which then rapidly self-organize to form the cords of seminiferous tubules that are perpendicular to the long axis of the testis, toroid like a donut, and uniform in thickness throughout its length. Testis cords are joined together at a point on their circumference corresponding to the rete testis.

Several studies in rodents have shown the requirement of the vascular endothelial cells in cord formation. Mesonephros contains the vascular plexus (MVP) which is rich in endothelial cells. Around E12.5 in the mice embryos, MVP breaks down presumably by the secretions from the developing testis, and the streams of endothelial cells migrate and partition the field of Sertoli and germ cells [28]. The proliferating Sertoli cells form stronger contacts around germ cell clusters, and the cords elongate. Both molecular and physical factors together aid in growing testis cords into toroid structures. As the space constrain, further extension of growing cords gets the complex "spaghetti" of testis tubules seen in adult testes. Migration of endothelial cells not only aids in partitioning of the cords but also leads to vascularization. The first blood vessels develop from the migrating endothelial cells originating from mesonephric vascular plexus and form the coelomic blood vessel or the aorta-gonad-mesonephros (AGM). Dependent on VEGF, the coelomic vessel branches and extend into the testis interstitium and tunica to connect into the rete testis. Little is known about the venous development during testis organogenesis. Venous vasculature seems to arise from the mesonephros probably following an already established arterial network.

At this stage, testis cords also develop an outer layer of peritubular myoid cells (PMCs) and deposit an extracellular matrix (ECM). This boundary helps to separate tubules from interstitium and acts as contractile tissue to facilitate sperm export—typically consisting of PMCs and ECM. The PMC lineage is thought to originate from the migrating mesonephric cells, however, the exact origin of these cells is not yet known.

In mice, trimming of testicular cords is known to occur by the yolk sac-derived macrophages that migrate along with the endothelial cells. Colonizing in the XY gonads, these macrophages engulf PMC and Sertoli cells; depletion of macrophages results in disorganized cord formation [29]. Whether macrophages colonize the developing human testis and contribute toward tubulogenesis is yet unknown.

Among the genes required for proper vascularization are the Desert hedgehog (Dhh), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), *Amh*, *Fgf9*, and neurotrophins. *Dhh* is expressed in pre-Sertoli cells shortly after *Sox9* activation. *Dhh* knockout mice display compromised development of

peritubular myoid cells (PMCs) and fetal Leydig cell (FLC) differentiation. In humans, mutations in *DHH* cause XY sex reversal [30]. Interestingly, developing testis of the *Dax1* knockout XY embryos also displays perturbed development of both FLCs and PMCs [31]. However, how DAX1 controls migration of PMCs and FLCs is yet unknown. The list of genes involved in these processes of testis development is listed in Table 1.1.

Leydig Cell Specification

Male gonad development requires the specification of Leydig cell; the primary source of steroid hormones. Decades of observations have suggested that Leydig cells found in fetal testes do not develop into those present in the adult but instead that the two cell types arise as two separate lineages with distinct functions and different cellular origins [32]. Origin of the fetal Leydig cell (FLC) lineage is debatable, and present evidence suggest that that FLCs stem from a shared pool of precursor cells. Based on profiling of single cells of XX and XY developing gonads, it is proposed that FLCs originate from *SF-1*-positive cells which are marked early on as pre-Sertoli cells. It appears that *SF-1*-positive cells that do not acquire *Sry* activation diverge toward the FLC lineage, while the *SF-1*-positive and SRY/SOX9-positive cells diverge to the Sertoli lineage [33]. In addition, a small proportion of FLCs appear to be recruited from a pool of *SF-1*-negative perivascular progenitor cells at the mesonephric-gonadal junction [34, 35]. As discussed above, *DHH* from the Sertoli cells also act as a paracrine trigger for FLC differentiation; Notch signaling also influences FLC differentiation [36]. Notably, Notch signaling does not appear to influence differentiated FLCs after E13.5 but rather acts to maintain a progenitor cell lineage.

FLCs produce androgens necessary for several aspects of sex differentiation which include development of the Wolffian ducts and masculinization of external genitalia, testicular descent, and perhaps sex-specific brain patterning [37, 38]. Lack of androgen biosynthesis or mutations in androgen receptor (*AR*) leads to feminization of the external genitalia and cryptorchidism in XY embryos, resulting in testicular feminization also called as androgen insensitivity syndrome. Androgen insensitivity syndrome (AIS) is a rare X-linked recessive disorder with an incidence of 1:20,000–64,000 male births. The affected males despite a normal XY karyotype and an intact *SRY* gene have blind vagina, absent uterus, and female adnexa with abdominal or inguinal testes. Partial androgen insensitivity syndrome (PAIS), also called Reifenstein syndrome, results in hypospadias and micropenis with gynecomastia. Most of the cases with AIS or PAIS have a mutation in *AR* gene and is a diagnostic feature of this condition.

Beyond *AR*, *SF-1* seems to be a critical gene whose mutations can cause sex reversal. As discussed above, *SF-1* is required to specify Sertoli cells and FLCs. Mutations in *SF-1* gene are observed in a spectrum of conditions ranging from complete XX and XY DSD to male infertility [39].

Lymphangiogenesis in the Testis

Lymphatic vessels are observed in the adult testis and are assumed to develop by lymphangiogenesis. This occurs relatively late in mouse gestation by ~E17, originating from a rich lymphatic network already established along the vasa deferentia and epididymis [32]. However, the genes required in this process are yet to be discovered. No information is available on development of lymphatic vessels in developing human gonads.

Germ Cell Sex Determination

Germ cells are the precursors of the sperm and oocytes, and as discussed above, they do not originate from the gonads but migrate and colonize the developing bipotential gonads. The primordial germ cells once in the gonads lose their migratory potential and get committed to gametogenic fate by expressing DAZL [40]. Like the somatic cells, the germ cells are also bipotential and can take any one of the two fates. Germ cell sex determination however occurs a little later: after E12.5 in the mouse and 10–12 weeks of gestation in humans. In the fetal ovary, germ cells enter meiosis and are committed to oogonia. In the fetal testis, they avoid entry into meiosis but undergo mitotic arrest and develop as spermatogonia. In the humans, there seems to be a requirement to maintain the appropriate number of germ cells during development majorly in the ovary but not the testis. It is shown that nearly 6–8% of germ cells in the human fetal ovary undergo cell death by apoptosis; this rate is accelerated in case of Turner syndrome resulting in an ovary without germ cells at birth [41].

While the somatic cell sex determination is cell autonomous, the decision of the germ cells to take one of the two sex fates is dependent on the somatic cells. Studies in mice and human developing gonads have shown that the entry of germ cells in meiosis is dependent on retinoic acid secreted by the somatic cells (of mesonephros). The *DAZL*-positive germ cells under the influence of retinoic acid express the pre-meiotic protein STRA8. Once the germ cells express STRA8, they get committed to enter meiosis and enter the oogonia fate. However, in the testis, germ cells are prevented from entering meiosis to enter the spermatogonia fate. This decision is dependent on the Sertoli cells. In both mice and humans, the *SOX9*-positive Sertoli cells abundantly produce the enzyme *CYP26B1* which actively degrades retinoic acid. In absence of retinoic signaling, the germ cells inside the tubules do not initiate the meiotic cascade, and the germ cells get arrested in mitosis phase committing to the spermatogonia phase [42]. Mice knockout for *Cyp26b1* though develop the testis normally; germ cells are not committed to spermatogonia fate and instead enter meiosis and die eventually [43].

Parallel to prevention of the meiotic program, germ cells also initiate a male program by activating the expression of RNA-binding protein *NANOS2*. *FGF9*

produced by the Sertoli cells aid in induction of *NANOS2* which prevents translation of gene products involved in oogenesis. Studies in knockout mice have shown that *NANOS2* is necessary and sufficient to promote aspects of male-specific germ cell identity [42]. While *NANOS2* is critical for identifying male germ line in the developing gonads, no mutations in these genes are yet reported to be associated with sex reversals or infertility. Mutation in *DAZL* is associated with severe spermatogenic failure and is associated with male infertility [44]. The key genes involved in germ cell sex determination are given in Table 1.1.

Antagonism of the Testis and Ovarian Pathways

For several years, it was believed that female development was the default program of the gonads until male development was actively switched on by *SRY.* However, the idea of ovarian development being a passive default option is challenged by the discovery of genes that actively promote ovarian development and suppress testicular program. These include *WNT4* and *RSPO1*. Both these genes not only are essential for ovarian development but are major repressors of testicular machinery described above. According to the current model, commitment of the bipotential gonad to testis or ovary fate is a consequence of antagonistic male and female pathways that compete to control the differentiation of supporting cell precursors (Fig. 1.2). According to this model, *SOX9* is a strong repressor of the ovarian fate genes like *Wnt4*, *Rspo1*, and *β-catenin* in the Sertoli cells. In absence of or reduced *Sox9* activation in XY gonads, the somatic cells instead of going toward the male pathway enter a female developmental program by expressing *Wnt4* and *Rspo1*; some of the germ cells enter meiosis, resulting in an ovo-testicular DSD. In contrast, absence of *Wnt4*, *Rspo1*, or *β-catenin* in the XX condition can result in stimulation of SOX9 resulting in differentiation of Sertoli cells and failure of germ cells to enter meiosis (oogonia fate). Also, the decisions taken by the gonadal cells during development need to be maintained in adult life. In adult testis and ovary, transdifferentiation can occur between Sertoli and granulosa cell fates, and this requires *DMRT1* and *FOXL2*, respectively. In mouse postnatal ovary, *FOXL2* represses the male pathway by suppressing the expression of *Sox9*, and when *Foxl2* is lost in adult ovaries, the granulosa cells transdifferentiate to Sertoli cells, and theca cells also begin to produce testosterone like the Leydig cells [19]. The antagonist signal for *FOXL2* in the adult testis is DMRT1. In mouse, DMRT1 promotes expression and maintenance of testicular genes such as *Sox9* and represses ovary-promoting genes such as *Foxl*2, *Wnt4*, and *Rspo1*. DMRT1 also antagonize the influence of retinoic acid, suggesting that maintenance of testis or ovary fate is an active process in adult life. While these studies in mouse are highly conclusive, the roles of *DMRT1* and *FOXL2* in maintenance of adult human gonadal fate are hitherto unknown. Human females carrying mutations in *FOXL2* gene develop blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) and are prone to premature ovarian failure, but do not carry the overt sex reversal phenotype as observed in mouse. Nevertheless, the

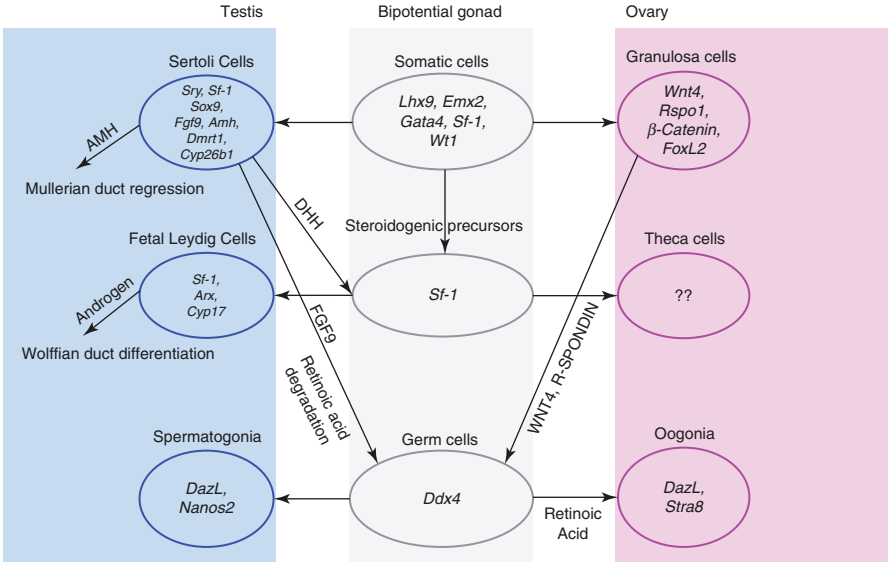

Fig. 1.2 Gonadal sex determination is a choice between two mutually opposing fates. The genital ridges contain bipotential precursor somaic and germ cells. *Lhx9, Emx2, Gata4, Sf-1* and *Wt1* are required for somatic cell development. When the somatic cells initiate *Sry-Sox9-Fgf9* expression, they get committed to the Sertoli cell fate and direct the testis differentiation (blue box). *Dmrt1* is required for maintaining Sertoli cell fate. Sertoli cells secrete DHH and trigger the differentiation of *Sf-1*-positive (*Sox9*-negative) steroidogenic precursors to fetal Leydig cells. Secretion of FGF9 and the degradation of retinoic acid by CYP26B1 from Sertoli cells promote *Ddx4* and *DazL* positive germ cell to differentate to *Nanos2* positive spermatogonia. Secretion of AMH by Sertoli cells regresses the Mullerian duct, and fetal Leydig cells secrete testosterone (androgen) for differentiation of the Wolffian ducts. In absence of *Sry*, the somatic cell fate switches to the granulosa cell fate under the influence of *Wnt4*, *Rspo1*, and *ß-Catenin*, leading to ovarian differentiation (pink box). *FoxL2* is required to maintain granulosa cell fate. The granulosa cells secrete WNT4 and R-SPONDIN, which along with retinoic acid induces *Stra8* in the *Ddx4* and *DazL* positive germ cells directing toward oogonia fate. The triggers for theca cell differentation in ovary are not understood. In absence of both AMH and androgens in the female gonads, the Wolffian ducts regress, and Mullerian ducts proliferate

plasticity of the human gonad is evident from recent studies in human developing gonads (second trimester fetus) where reducing the expression of *DMRT1* causes compromised Sertoli cell functions leading to testicular dysgenesis and induction of the ovarian marker FOXL2 [45].

Summary

Testis development involves the initial specification of Sertoli cells by expression of *SRY-SOX9-FGF9* cascade that triggers organization of seminiferous tubules, differentiation of Leydig and peritubular myoid cells, and finally vascularization. At

Table 1.2 Gene mutations involved in DSDs

Karyotype	External phenotype	Gonadal phenotype	Gene mutations
46,XY	Female	Dysgenetic gonad	No *SRY*, mutations in *SRY*, *GADD45G*, *SOX9*, *DAX1* duplication
46,XY	Female	Ovotestis	*SRY* translocation
46,XY	Female	Inguinal testis	Mutations in androgen receptor and *Cyp17A*
46,XY	Male, ambiguous	Ovotestis	Mutations in *NR5A1*, *CBX2*, *DHH*, *MAP3K1* Deletions of *DMRT1* or *EMX2* Duplication of *DAX1*
46,XY	Male	Persistence of Mullerian ducts	*AMH*, *AMHR*
46,XX	Male, ambiguous	Dysgenetic gonad or ovotestis	*DAX1* duplication *SRY* translocation on X chromosome Duplication of *SOX10* Mutations in *RSPO1* and *WNT4*
46,XX	Ambiguous	Ovary with congenital adrenal hyperplasia	CAH, mutation in Cyp17A
45,X	Female	Dysgenetic gonad or reminiscent ovary	Loss of X chromosome
46XX	Female	Mullerian agenesis	Activating mutations in *AMH*

the same time, germ cells inhibit meiosis by the virtue of Sertoli cells degrading retinoic acid and entering spermatogonia fate. Decision of the bipotential gonad to form the testis is however not irreversible and requires maintenance by DMRT1. In absence of the key genetic players involved in Sertoli cell development and maintenance, the gonad can be compromised, and the cells can take an alternate (ovarian) fate. This knowledge has helped us in understanding the etiology of various DSDs (Table 1.2). While the picture of the genetic network required for testis development and sex differentiation is clear, we are unable to explain the etiology of DSD in most of the cases. A better understanding of the players involved in the process of testis differentiation is required for evolving strategies for diagnosis and management of DSDs.

Review Criteria

- Title of the chapter "The Molecular Genetics of Testis Determination" was searched in PUBMED—**7824** results
- Title of the chapter was searched in PUBMED under Review only filter—**283** results
- Out of 283 reviews, only relevant to human and mouse were screened—138 results
- Full reviews and back references were read—**138** and **10**
- References cited in present chapter—**45**

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Chapter 2
Molecular Regulation of Sperm Production Cascade

Meghali Joshi and Rajender Singh

Key Points

- The process of spermatogenesis involves the proliferation of spermatogonial stem cells (SSCs) and their eventual differentiation into sperm post-meiosis.
- The proliferation and differentiation of SSCs are regulated by numerous intrinsic and extrinsic factors.
- Glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2) are produced by the Sertoli cells, which act as an extrinsic factors for SSC proliferation and differentiation.
- Retinoic acid initiates the process of differentiation of undifferentiated spermatogonia.
- The differentiation process an increase in the expression of genes involved in differentiation such as Sohlh1, Sohlh2, Kit, Ccnd2, and Sall4.
- Meiosis in testis is initiated by retinoic acid by activating Stra8.
- During the process of spermiogenesis, axoneme formation, manchette formation, and chromatin remodelling occur.
- Mutations in the genes involved in the process of spermiogenesis may result in defects in manchette formation, flagellar motility, and chromatin condensation.

M. Joshi · R. Singh (✉)
Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh, India
e-mail: rajender_singh@cdri.res.in

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Introduction

Spermatogenesis is a complex process, which takes place in the testes within the seminiferous tubules. Two types of cells reside within the seminiferous tubules: germ cells and Sertoli cells. For continuous production of spermatozoa throughout life, the Sertoli cells play a very important role in providing the microenviornment. In the interstitial space, the Leydig cells are present, which produce growth factors and testosterone. Cells such as peritubular myeloid cells surround the seminiferous tubules and provide structural support and growth factors and help in the movement of fluids and sperm through the tubule lumen [1]. During the process of spermatogenesis, spermatogonial stem cells either mitotically proliferate to maintain their population or enter differentiation to ultimately produce spermatozoa. This whole process is divided into three major phases: the first is self-renewal of spermatogonial stem cells, the second is meiotic divisions of spermatocytes to give rise to haploid round spermatids, and the third is the spermiogenesis process where round spermatids differentiate into mature haploid spermatozoa [1].

Several studies have reported genetic abnormalities leading to male infertility. Infertility affects ~15% of couples, and half of these cases are associated with male-related factors [2]. The cause of infertility could be due to no sperm production (azoospermia), low sperm count (oligozoospermia), low sperm motility (asthenozoospermia), abnormal morphology (teratozoospermia), or a combination of these. Azoospermia genetic causes include Y chromosome microdeletions, chromosomal abnormalities, and specific mutations or deletions of genes present on the sex chromosomes and autosomes [3, 4]. It was reported that mutations in the DAZ gene (Yq11.23) caused human male infertility that ranged from oligozoospermia to azoospermia [5, 6]. In another report, it was shown that mutations in the RBMY gene (Yq11.223) could lead to meiotic arrest, ultimately causing azoospermia [6, 7]. The azoospermia factor (AZF) region on the Y chromosome has been the most commonly studied region in male infertility [8, 9].

Similarly, other mutations have been reported in male infertility. For example, mutations in human SYCP3 gene (12q23) were reported in male fertility, which resulted in arrest at the meiotic stage [10]. Missense and splicing mutations in the germ line-specific gene KLHL10 (17q21) have been reported in oligozoospermia [11]. This gene is essential for spermiogenesis and acts in a dose-sensitive manner [11]. Aurora kinase C (AURKC) at 19q13 is highly expressed in testis [12, 13] and is involved in mitosis, meiosis, and cytokinesis [14, 15]. Mutations in the AURKC gene caused macrozoospermia (large-headed polyploidy spermatozoa) and male infertility [16, 17]. Similarly, mutations in the HSF2 (heat-shock transcription factor) gene are involved in human idiopathic azoospermia [18]. In a recent study, we reported the association of PSA/KLK3 mutations with male infertility [19].

In this chapter, we discuss the important genes involved in spermatogonial stem cell self-renewal and differentiation and genes involved in meiosis and spermiogenesis. Further, we also present details of the mutations in these genes and their association with impaired spermatogenesis and male infertility.

Spermatogonial Stem Cells (SSCs)

During embryonic development, the PGCs migrate to the genital ridge where they differentiate into prospermatogonia and enter mitotic arrest by 13.5–15.5 dpc (days post coitum) [20]. After birth, male germ cells enter mitosis. Subsequently, a subset of them enters into the differentiation process, leading to meiosis and spermiogenesis. Continuous production of sperm throughout life involves the self-renewing of the pool of precursor cells called as spermatogonial stem cells (SSCs) [21, 22]. At postnatal day 3–4, spermatogonial population is heterogeneous having undifferentiated (A_{undiff}) and differentiated (A_{diff}) spermatogonia [23]. A minor fraction of the A_{undiff} spermatogonia consists of the spermatogonial stem cell (SSC) pool, which is estimated to be 1 in 3000 cells in adult mouse testis [24]. Based on the morphological analysis of mice testis, SSCs (A_{single}) and committed progenitor spermatogonia (A_{paired} and $A_{aligned}$) are collectively described as undifferentiated A spermatogonia [25].

The two most important properties of the SSCs are self-renewal and differentiation. The division of SSCs either gives rise to similar cells for maintaining their own population or produces progenitor spermatogonia for further differentiation. These differentiating spermatogonia have relatively large tubular connection, called intercellular or cytoplasmic bridge that results from incomplete cytokinesis [26]. Single spermatogonia are termed A_{single}, while those connected by an intercellular bridge are termed A_{paired}. The commitment to enter meiosis is made with the transition of A_{undiff} into A_{diff} spermatogonia, and the first differentiating spermatogonia are termed type A_1. A_1 cells divide by mitosis and form A_2 cells, which in turn divide and create A_3, a division of which generates A_4 spermatogonia. Next, two mitotic divisions form intermediate and B spermatogonia [27] (Fig. 2.1). In the section below, we have discussed the extrinsic and intrinsic factors involved in the SSC self-renewal and differentiation and the mechanisms of gene regulation.

Extrinsic Factors

Glial cell line-derived neurotrophic factor (GDNF) was first identified to play a crucial role in the SSC self-renewal by both in vivo and in vitro studies. GDNF is secreted by the Sertoli cells in the seminiferous tubules. GDNF regulates undifferentiated spermatogonia in a dosage-dependent manner [28]. Gene-targeted mice having GDNF-null allele showed loss of undifferentiated spermatogonia, whereas mice overexpressing GDNF showed accumulation of undifferentiated spermatogonia [28]. GDNF is known to regulate self-renewal via its multicomponent receptor complex consisting of GFRA1 and RET as the depletion of both these receptors resulted in a similar phenotype as reported in GDNF ablation [29]. GDNF promotes proliferation of spermatogonial stem cells by activating different signaling pathways. It has been reported in in vitro culture and transplantation assay that GDNF activates

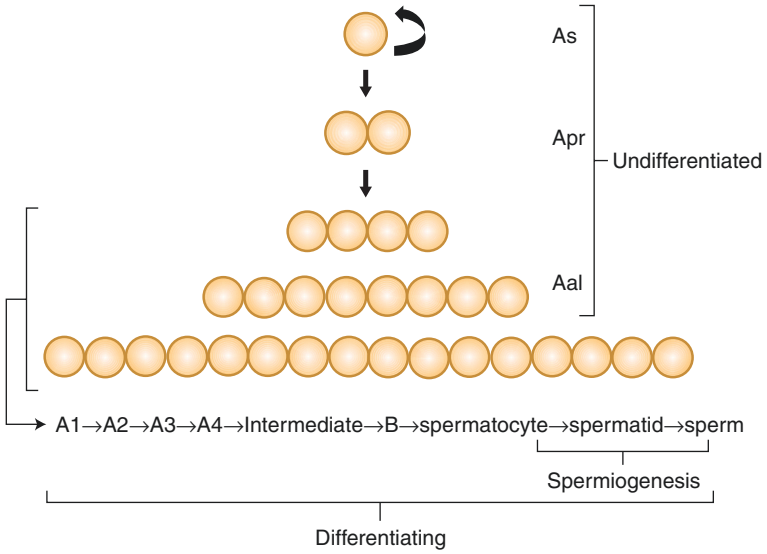

Fig. 2.1 Developmental stages of spermatogenesis. A_{single} (A_s) gives rise to A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}), which get differentiated into type B spermatogonia, and the latter gives rise to spermatocytes. Spermatids are generated upon completion of meiosis, which ultimately differentiate into sperm

PI3K-AKT signaling pathway [30–32]. GDNF also activates Src family kinase (SFK) signaling. This signaling pathway promotes self-renewal, partly through AKT signaling. It has been reported that in the presence of AKT inhibitor, the proliferation of SSCs completely disrupts, while SFK inhibitor disrupts it partly. This shows a dominant role of AKT signaling over SFK in regulating the self-renewal of SSCs. He et al. (2008) found that the RAS/ERK1/2 signaling pathway is also activated by the GDNF. This pathway regulates self-renewal and proliferation, via increasing the phosphorylation of CREB/activating transcription factor 1 family member and by upregulating c-FOS transcription factor expression [33]. Another important extrinsic factor is fibroblast growth factor 2 (FGF2), which along with GDNF promotes self-renewal of SSCs. FGF2 promotes proliferation by activating the MAP2K1 pathway. Three transcription factors (Bcl6b, ETV5, and Lhx1) were downregulated by MAP2K1 inactivation showing that they act downstream of the FGF2 pathway [34].

Intrinsic Factors

Six genes (Bcl6b, Etv5, Lhx 1, Egr2/3, and Tspan8) are known to play important roles in self-renewal and proliferation of the SSCs. These genes showed down-regulation in GDNF-deficient culture medium and upregulation in GDNF-treated culture medium [35]. Bcl6b knockdown by siRNA and knockout experiments resulted in decrease in viability of cells and increase in apoptosis showing that bcl6b plays

an important role in SSC renewal and maintenance [35]. Similar phenotype, i.e., reduced SSC numbers, was seen when Etv5 gene was knocked down in THY^{+1} cultured spermatogonial cells by Etv5-specific siRNA [36]. A microarray study reported that siRNA depletion of Etv5 leads to the downregulation of SSC proliferation genes Bcl6b, Lhx1, and Brachyury [37]. Another important transcription factor, ID4, an inhibitor of DNA-binding protein 4, is induced by GDNF [38]. ID4 has been known to play a role in SSC maintenance as ID4 knockout study has shown age-dependent germ cell loss in mice [38]. Another GDNF-induced gene, Nanos2, which is a zinc finger RNA-binding protein, plays an important role in SSC maintenance. The knockout of Nanos2 in mouse showed germ cell loss, while Nanos2 overexpression resulted in the accumulation of promyelocytic leukemia zinc finger (PLZF+) spermatogonia in mice [39]. Spermatogonial stem cell maintenance and self-renewal can be regulated independent of GDNF. PLZF uniquely expresses in undifferentiated spermatogonia and was first reported as an important maintenance factor. PlZF is encoded by Zbtb16 gene. A nonsense mutation in Zbtb16 caused infertility and germ cell loss in mice [40]. PLZF was reported to inhibit spermatogonial differentiation by binding to the c-kit promoter region and repressing its expression [41]. Additionally, Hobbs et al. (2012) showed that SALL4 inhibits PLZF and increase in SALL4 expression results in c-Kit transcription. Thus, mutual effects of SALL4 and PLZF maintain the balance between SSC self-renewal and differentiation [42].

Taf4b, which is a transcription factor expressed in both spermatogonia and Sertoli cells, has been shown to play a crucial role in SSC maintenance as Taf4b loss in mice induced age-dependent germ cell loss [43]. Moreover, transplantation of normal SSCs into Taf4b-depleted testis resulted in normal spermatogenesis, suggesting that Taf4b behaves in a cell autonomous manner. FOXO1 has been known to play an essential role in SSC maintenance. It has been reported that FOXO1-depleted mice testes have similar defects of SSC maintenance as reported in other maintenance factors (e.g., PLZF, Taf4b, Etv5), i.e., age-dependent decline in spermatogenesis and germ cell loss [44]. Recently, Shinohara et al. (2016) reported that Myc is an important transcription factor, which maintains the balance between SSC self-renewal and spermatogonial differentiation as overexpression of Myc increases self-renewal, whereas disruption of Max, a myc-binding partner, leads to meiotic initiation [45]. Furthermore, they found that myc/mycn double knockout SSCs not only impair self-renewal but also affect glycolysis. The inhibition of glycolysis resulted in decreased SSC division, while chemical stimulation of glycolysis restored SSC self-renewal [45] (Fig. 2.2).

Spermatogonial Differentiation

The differentiation process starts from the transition of A_{al} into A_1 spermatogonia, and this process is strictly time regulated. The mechanism by which spermatogonial differentiation takes place is still to be explored. The process of differentiation into

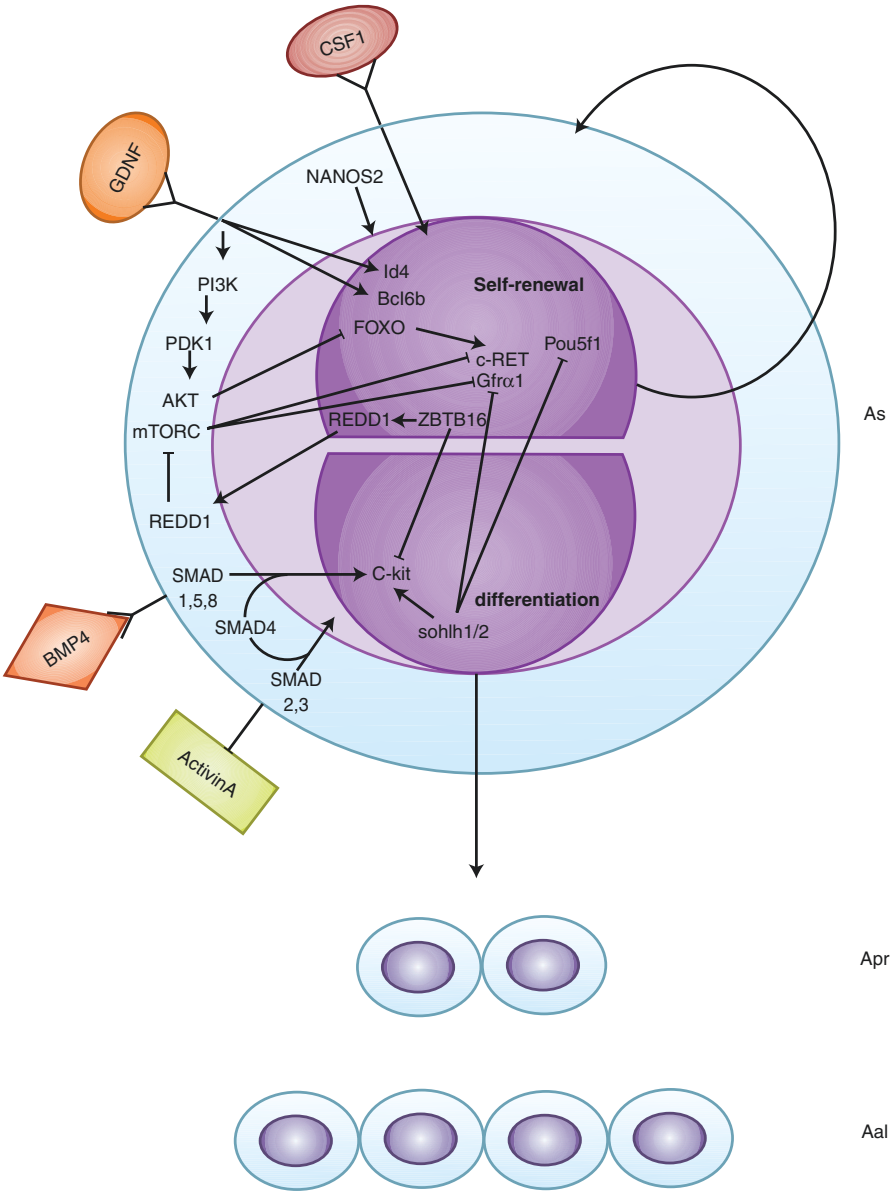

Fig. 2.2 Important pathways involved in self-renewal and differentiation process in rodent spermatogenesis

A_1 spermatogonia is initiated by retinoic acid. It has been reported that in vitamin A-deficient animals, undifferentiated spermatogonia fail to differentiate into A_1 spermatogonia, illustrating the importance of retinoic acid in spermatogonial differentiation [46–49]. Retinoic acid is produced by two successive reactions. First

retinol is converted into retinal by retinol dehydrogenases, and second retinal is converted into retinoic acid by retinaldehyde dehydrogenases. The undifferentiated spermatogonia failed to differentiate into A_1 spermatogonia in mice having conditional deletion of retinol dehydrogenase 10 (Rdh10) in Sertoli plus germ cells [50]. Interestingly, this defect was observed till the first wave of spermatogenesis in young mice (age, <7 week), after which, KO males showed normal fertility and testis histology suggesting that conversion of retinol to retinal is performed by another retinol dehydrogenase in adult mice. Moreover, conditional deletion of three retinaldehyde dehydrogenases (Aldh1a1–3, previously termed Raldh1–3) in mouse Sertoli cells [51] resulted in the loss of spermatogonial differentiation; however, treating mice with RA or a retinoic acid receptor (RAR) A selective agonist reinitiates spermatogenesis. It was found that the deletion of Sohlh1 and Sohlh2 blocks and deletion of Sox3 impair spermatogonial differentiation with defects in Sox3 KO testes being severe during the first wave of spermatogenesis that improved over time as the mice age [36, 52–55]. At the time of spermatogonial differentiation, the undifferentiated spermatogonia downregulate the genes involved in self-renewal and upregulate the genes involved in differentiation such as Sohlh1 [36], Sohlh2 [56], Stra8 [57], Kit [58], Ccnd2 [59], and Sall4 [60]. The tyrosine kinase receptor c-KIT expression is induced upon differentiation of A_{al} into A_1 spermatogonia [58]. In addition, it has been observed that A_{al} spermatogonia fail to differentiate into A_1 spermatogonia in mice germ cells having heterozygous mutation in the c-Kit white spotting (w) locus or mutation in its ligand stem cell factor (SCF) [61, 62]. Another gene CCND2 (Cyclin D2) has similar expression pattern as c-KIT, i.e., its expression is induced during the A_{al}-A_1 transition and further maintained till the spermatocyte level, which reflects its requirement during meiosis [59].

Spermatocytes and Meiosis

Meiosis is a crucial process in spermatogenesis that initiates when type B spermatogonia (diploid) divide into pre-leptotene spermatocytes and further differentiate to give rise to haploid spermatids. In this process, one round of DNA duplication and two consecutive rounds of chromosome segregation meiosis I and meiosis II give rise to haploid round spermatids. Meiosis initiates through retinoic acid by induction of Stra8 (stimulated by retinoic acid gene 8). It has been reported that germ cells failed to enter meiotic prophase stage in Stra8-deficient mice testes [63]. However, the early mitotic divisions of spermatogonia remain undisturbed. Therefore, Stra8 is required for the initiation of meiosis in spermatogenesis [63].

During the first meiotic division, chiasmata are formed for the proper positioning and subsequent segregation of the homologous chromosomes. Formation of chiasmata is a crucial process in which DNA double-strand breaks (DSBs), homologue recognition, and meiotic recombination take place. During synapsis of the homologous chromosomes, zipper-like structure is formed named synaptonemal complex (SC) [64]. In leptotene spermatocytes, synaptonemal complex forms fibrous axial

element alongside the sister chromatids of homologous chromosomes. The three major components of the axial elements are SYCP1, SYCP2, and SYCP3. SYCP1 are transverse elements in the synaptonemal complex that forms bridges between homologous chromosomes [65], whereas SYCP2 and SYCP3 are present on sister chromatids and act as lateral elements. In order to investigate the functions of these three synaptonemal proteins Sycp1, Sycp2, and Sycp3 in mammals, knockout mouse models have been developed [65–67]. In Sycp2 mutant male mice, meiosis fails as Sycp2 mutant protein prevents binding of Sycp3 to the lateral elements [67]. Moreover, this mutation in Sycp2 gene leads to apoptosis of zygotene spermatocytes. In Sycp2- and Sycp3-deficient male mice, homologous chromosomes fail to form synapse [66]. In Sycp1-deficient mice, most of the primary spermatocytes were arrested at the pachytene stage and later enter apoptosis [65].

Hormad1 is present at the unsynapsed chromosome axis and is involved in the double-strand break and synaptonemal complex formation during meiosis [68–70]. Hormad1 is essential for normal mammalian gametogenesis. Hormad1-deficient male mice were infertile and showed meiotic arrest in the early pachytene stage [70]. However, Hormad1-negative testes do not have any effect on the localization of synaptonemal complex proteins like Sycp2 and Sycp3, but it disrupts homologous chromosome pairing. In addition to this, double-strand break formation and early recombination events were disrupted in Hormad-deficient mice testes [70].

When homologous chromosomes pair together, the meiosis-specific HORMA domain proteins, Hormad1 and Hormad2, are removed from the chromosome axis by checkpoint protein TRIP13 [68]. These HORMA domain proteins remain present at the unpaired chromosome axis and recruit the kinase ATR [71, 72], which along with other proteins like BRCA1 and γH2AX leads to the silencing of transcription from unpaired chromosomal regions. This process of silencing is referred to as meiotic silencing [73, 74]. Usually, X and Y sex chromosomes remain unsynapsed except pseudo-autosomal regions (PARs) and undergo meiotic silencing. This leads to the formation of the XY body, in which the sex chromosomes are silenced by ATR, BRCA1, and γH2AX [75, 76]. Nevertheless, in case of extensive autosomal asynapsis, these proteins fail to silence sex chromosomes and lead to stage IV pachytene arrest. Royo et al. (2010) have shown that the lack of timely silencing of the Y chromosome genes Zfy1 and Zfy2 leads to the apoptosis of spermatocytes in mouse [73]. It has been observed that stimulation of ATR activity requires direct interaction with an ATR activation domain (AAD)-containing partner. Investigators later found this AAD-containing partner to be the DNA damage and checkpoint protein TOPBP1 [77]. In Topbp1 cKO male mice, defective recombination and synaptonemal complex formation were observed [77].

During meiosis, DSB formation and repair takes place which employs several DNA damage proteins. Several studies have been conducted identifying their functions during meiosis [78–81]. It has been observed that disruption of these proteins leads to stage IV pachytene arrest [82]. Spermatocytes of different mouse mutants show different cytological endpoints, but they all are eliminated at the same stage of spermatogenesis [83]. For instance, Atm-deficient spermatocytes reach till the leptotene stage [84, 85], while Dmc−/− [83, 86], Spo11−/− [83, 87], and Msh5−/−

spermatocytes reach the zygotene stage [88]. In Sycp1- [65] and Smc1β-deficient [89] spermatocytes, synapsis is disturbed, arresting at the pachytene stage before undergoing apoptosis [90]. But all the spermatocytes are eliminated at stage IV. Therefore, both the cytological endpoint and the elimination of spermatocytes from the seminiferous epithelium at stage IV seem to be two separate events.

Spermatids and Spermiogenesis

The completion of the second meiotic division in the seminiferous tubule gives rise to haploid cells called spermatids. Initially, spermatids are round and immotile cell, which later differentiate into specialized and motile spermatozoa. This process of differentiation is called spermiogenesis. During spermiogenesis, these cells undergo significant morphological and cytological changes. Morphologically, these round-shaped spermatids develop distinct head, midpiece, and tail regions. Cytologically, they undergo chromatin remodelling, most of the cytoplasm is removed, and acrosome is formed.

Axoneme

The sperm tail or flagellum starts to develop in round spermatids during step 1 of spermiogenesis [1] and is divided into the middle, principal, and end pieces. The flagellum develops from a centriole, which is present at one pole of the round spermatid and is composed of a cytoskeletal structure known as the axoneme [91]. Several mutational studies have shown that defects in axoneme formation lead to male infertility [92]. Defects in axoneme formation are related with motility loss. Spag17 encodes a protein which is present in the axoneme central pair complex [93]. Deletion of Spag17 has been associated with infertility caused by defects in sperm motility [93]. Moreover, protein transport and manchette microtubules were disrupted, and sperm collected from the cauda epididymis were immotile and had defects in tail and head morphology. In Sox30−/− testes, germ cell development arrests during the post-meiotic stage [94]. Additionally, axoneme development was aberrant with no elongated spermatid nor spermatozoa produced. The deletions of HOP, SPAG6, and TEKSTIN-T, which are involved in axoneme formation, lead to motility defects [95–97].

Manchette Formation and Cytoplasmic Exclusion

During nuclear elongation, a transient skirt-like structure called manchette is formed [98] (Fig. 2.3). Manchette consists of microtubules and actin filaments and plays a crucial role in shaping the nucleus and the sperm head [98]. There are

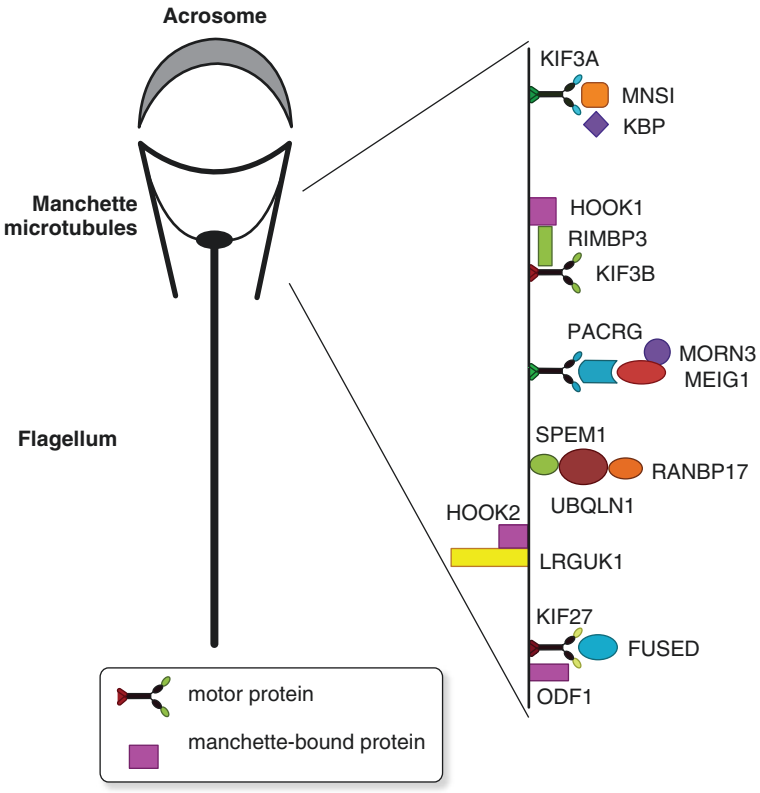

Fig. 2.3 A model illustrating the manchette formation and protein complexes associated with it during spermatid elongation

numerous genes involved in manchette formation, and deletions of these genes have been reported to result in defective manchette formation [99–101]. The deletion of Azh (abnormal spermatozoon head) in mice results in defective manchette formation with bend and coiled tails and decapitated sperm [99]. HOOK1 is a gene which helps in connecting the manchette to the nucleus [99, 100]. Mutation of Hook1 has defects in flagellar motility and sperm decapacitation [99]. Leucine-rich repeats and guanylate kinase-domain containing isoform 1 (LRGUK-1) are required for basal body attachment to the plasma membrane, shaping of sperm head, and axoneme formation [101]. The deletion of LRGUK-1 is associated with abnormal manchette formation and movement and defects in the initiation of axoneme growth from the basal body [101]. LRGUK-1 is reported to function in partnership with other proteins like Rab-interacting molecule-binding protein (RIMBP)-3, kinesin light chain (KLC)-3, and members of the HOOK family of proteins (HOOK-1–3). All these are localized in manchette and are responsible for intracellular protein transport [102].

Cytoplasmic exclusion is a crucial process in spermatogenesis, ensuring the development of compact and slender spermatozoa. A protein encoded by the gene spermatid maturation 1 (Spem1) is expressed in the cytoplasm of steps 14–16 elongated spermatids in the mouse testes [103]. The loss of Spem1 results in the retention of cytoplasmic remnants in the head and neck region, obstructing straightening and stretching of sperm head and neck, leading to sperm deformation and male infertility [103]. In another study, it was found that Repro32-null mice were infertile and had immotile sperm and low epididymal sperm concentration with sperm head defects (Geyer et al. 2009). Normally, in mice, the capping protein (actin filament) muscle Z-line, alpha 3 (CAPZA3), interacts with F-actin and plays a role in cytoplasm removal. It was identified that Repro32 null mice consist of a mutation in CAPZA3 gene, leading to abnormal removal of the cytoplasm [104].

Chromatin Remodelling

Chromatin remodelling is the most important phase of spermiogenesis in which histones are replaced by sperm-specific protamines to form a highly compact chromatin structure of mature sperm. This leads to nuclear condensation and transcriptional silencing. In mammals, firstly transition nuclear proteins (Tnps) are incorporated in place of histones; subsequently Tnps are replaced by protamines (Prms) [105]. It has been reported that for the incorporation of Tnp [106, 107], histones are modified by hyperacetylation [108, 109] and phosphorylation for their own removal. Mutation in the genes involved in histone acetylation and phosphorylation like pygo2 or Sstk results in defects in chromatin condensation, leading to male infertility [106, 107]. Tnp1-deficient mice showed more severe spermatogenic defects when compared to Tnp2-deficient mice. This is due to a higher expression of TP1 in wild mouse testes [105]. Further, proteins which are required for the transcription of Tnps and Prms, such as CREM and TRF2, were disrupted that resulted in defective chromatin condensation and male infertility [110, 111]. The post-translational modification of Prms is very important for successful incorporation into chromatin. It has been reported that the loss of kinase CAMK4, which is involved in phosphorylation of PRM2, inhibits its phosphorylation preventing its incorporation in chromatin, leading to male infertility [112]. It has been reported that chromodomain helicase DNA-binding protein 5 (Chd5) is the master regulator of histone-to-protamine chromatin remodelling and that the loss of CHD5 is associated with defective chromatin compaction and male infertility in mice [113]. Sly gene is encoded by mouse Y chromosome and is expressed in the post-meiotic germ cells only. It has been reported that Sly binds to the promoter of genes present on the sex chromosomes and autosomes which are involved in chromatin regulation. The loss of Sly resulted in altered chromatin remodelling having impact on histone to protamine exchange and ultimately affects the sperm genome integrity [114].

Conclusion and Future Perspective

The process of spermatogenesis, which initiates from the SSCs, is highly orchestrated and complicated. SSCs are the origin of spermatogenesis; thus, any defect in SSC self-renewal and differentiation can result in severe infertility. Several animal studies mentioned in this chapter have provided major insights into the molecular control of spermatogenesis. The proliferation and differentiation of the SSCs are regulated intrinsically by stem cells themselves and extrinsically by the factors produced by the surrounding Sertoli cells. GDNF is an important extrinsic factor produced by Sertoli cells. GDNF along with its multicomponent receptor complex consisting of GFRA1 and RET is known to regulate self-renewal of SSCs. GDNF promotes proliferation by activating several signaling pathways like PI3K-AKT, Src family kinase (SFK), and RAS/ERK1/2 signaling. Another extrinsic factor FGF2 promotes self-renewal by activating MAP2K1 signaling pathway. Microarray studies have identified several genes important for SSC self-renewal, such as Bcl6b, Etv5, Lhx1, Egr2/3, Tspan8, and Brachyury. Numerous knockdown studies have identified critical players (ID4, Nanos, Zbtb16, Taf4b, Foxo1, and Myc) in the proliferation of SSCs.

The next step involves the differentiation of undifferentiated spermatogonia. This step is initiated by retinoic acid. Deletions of genes (Rdh10, Aldh1a1–3) involved in retinoic acid synthesis result in the loss of spermatogonial differentiation. During differentiation, undifferentiated spermatogonia downregulate the self-renewal genes and upregulate genes which are involved in differentiation such as Sohlh1, Sohlh2, Stra8, Kit, Ccnd2, and Sall4. Further, differentiated spermatogonia undergo meiosis I, forming diploid spermatocyte, and meiosis II to give rise to haploid round spermatid. Meiosis is initiated by retinoic acid by activating Stra8. During meiosis, DNA double-strand breaks (DSBs), homologue recognition, and meiotic recombination take place. Several proteins are involved in this process, for example, synaptonemal complex proteins (Sycp1–3), meiosis-specific HORMA domain proteins (hormad1 and hormad2), and DNA damage proteins (Dmc, Spo11, Msh5). Sex chromosome inactivation is an important step in meiosis. Genes involved in the process of meiotic silencing are Brca1, γH2AX, ATR, TOPBP1, Zfy1, and Zfy2. Mutant mice having deletions in these genes display arrest at different stages of spermatogenesis.

The final step in male germ cell maturation is spermiogenesis in which round spermatids are differentiated into specialized and motile spermatozoa. During this process, axoneme formation, manchette formation, and chromatin remodelling occur. Mutations in the genes involved in axoneme formation (Spag17, Sox30, Spag6, Hop, Tekstin-T) result in motility defects. Manchette formation and cytoplasm removal are the important processes taking place during spermiogenesis. The deletion of genes involved in the manchette formation (Azh, hook1, lrguk1) results in defective manchette formation, defects in flagellar motility, and sperm decapacitation. Similarly, the deletion in Spem1 and Repro32 genes resulted in abnormal removal of cytoplasm from the head and neck region of developing spermatozoa.

The disruption of genes involved in chromatin remodelling, Pygo2, Sstk, Tnp1, Prms, Camk4, Chd5, and Sly, results in defective chromatin condensation, leading to male infertility.

The advent of whole genome studies has accelerated research into the identification of the causative genes for male infertility. In a recent study, whole exome sequencing was performed on 78 patients, identifying mutations in the genes DNAH1, CFAP43, and CFAP44 [115]. This was followed by CRISPER-Cas9-mediated knockout of Cfap43 and Cfap44 genes, showing that knockout male mice were infertile with severe flagellar defects. Similarly, a mutation in the gene TDRD9 was identified using whole genome genotyping and sequencing [116]. The mutations in this gene resulted in arrest in sperm production. Mouse knockout studies have provided a plethora of data and have been instrumental in the identification of infertility candidate genes for the last several decades. Nevertheless, occasional and random defects in spermatogenesis in human infertility provide us with natural models of infertility. In order to elucidate the complex molecular control of spermatogenesis, modern whole genome studies that focus on the identification of novel genes involved in infertility need to be undertaken in human infertility cases. This would accelerate the identification of the causative genes and also identify the targets for its treatment.

Review Criteria

- PubMed search for spermatogonial stem cells, spermatogonial differentiation, mice, and human ($n = 204$)
  - Articles screened through title and abstract

    Studies in other animals excluded ($n = 96$)
    Excluded articles related to stem cells in other organs ($n = 32$)
  - There were 76 articles cited from this search
- Secondary PubMed search for spermatocytes, spermatids, molecular regulation, mice, and human ($n = 78$)
  - Articles screened through title and abstract

    Studies in other animals excluded ($n = 25$)
    Excluded articles which were not related to the keywords used ($n = 12$)
  - There were 41 articles cited from this search.

  A total of 117 articles were cited.

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Chapter 3
Spermatozoal Chromatin Structure: Role in Sperm Functions and Fertilization

Sara Marchiani, Lara Tamburrino, Monica Muratori, and Elisabetta Baldi

Key Points

- Chromatin compaction occurs during the last phases of spermiogenesis and epididymal transit. During chromatin compaction process, about 90% of histones are replaced by protamines.
- Chromatin compaction is essential to protect sperm DNA from external insults during its travel to the oocyte.
- Improper chromatin compaction is a marker of abnormal spermiogenesis and is related to lower reproductive outcomes, affecting sperm ability to fertilize and embryo development.
- Sperm chromatin status can be evaluated by simple tests whose results are related to assisted reproduction outcomes.

Introduction

Following the onset of puberty, the process of spermatogenesis is appointed to originate and maintain the daily production of fully differentiated spermatozoa throughout the reproductive lifespan of males. Such process is characterized by a myriad of

S. Marchiani
Andrologia, Endocrinologia femminile e Incongruenza di genere- Dipartimento Materno Infantile, Azienda Ospedaliero Universitaria Careggi, Florence, Italy

L. Tamburrino · E. Baldi (✉)
Experimental and Clinical Medicine, University of Florence, Florence, Italy
e-mail: elisabetta.baldi@unifi.it

M. Muratori
Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy

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changes that lead to the generation, from an immature diploid spermatogonium to four haploid spermatozoa in the testis. At the end of the maturation process, spermatozoa are characterized by an extreme compacted DNA with respect to the nucleus of somatic cells. To reach such compaction, a dramatic reorganization occurs in developing spermatids where the vast majority of somatic histones are replaced by small basic proteins, called protamines 1 and 2, leading to a highly condensed and packaged chromatin. After spermiation, spermatozoa will complete their maturation during the epididymal transit, where they acquire the ability to move progressively and terminate the process of chromatin compaction.

Chromatin compaction is required to protect paternal genome during the travel of spermatozoa to the oocyte and makes the spermatozoon able to carry out its main function, which is to be the vehicle that delivers the haploid paternal genome to the oocyte allowing the onset of a new life.

The importance of chromatin packaging for sperm functions is demonstrated by the occurrence of alterations in protamine and histone content, as well as protamine 1/protamine 2 ratio in infertile men. Such alterations have a negative impact also on the success of assisted reproduction techniques (ARTs, Table 3.1), which represents the option with the highest chance of achieving pregnancy for most of infertile subjects. The paternal genome is considered to be inactive, and the role of retained histones emerged in recent studies demonstrating that sperm histones inherited by the embryo deliver epigenetic markers involved in the activation of key genes of embryogenesis [1]. Consequently, the chromatin status of spermatozoa may affect

Table 3.1 Relationship between sperm chromatin status and ART outcomes according to the method used to detect chromatin maturity

Test		Relation with ART outcomes		References
Chromomycin A3	An index of protamination	Poor protamination	Reduced FR	[64, 83–86]
			Poor EQ	[68, 87, 88]
			Low PR	[64, 67, 89]
Aniline blue	An index of histone retention	Abnormal histone retention	Reduced FR	[68, 88]
			Poor EQ	[69]
			Low PR	[66, 69, 90]
Toluidine blue	An index of chromatin condensation	Poor chromatin condensation	Reduced FR	[91]
			Poor EQ	[92]
			Low LBR	[87]
P1/P2 ratio	An index of protamine content	Altered ratio	Reduced FR	[40, 60, 93]
			Poor EQ	[40, 93]
			Low PR	[40, 63, 94]
PRM1 and PRM2	A measure of protamine transcripts	Increased protamine mRNA	Higher FR and EQ	[79]
		Normal protamine mRNA ratio	Higher FR	[62]
H2B to protamine ratio	An index of histone replacement	Higher ratio	Poor EQ	[95]

FR fertilization rate, *EQ* embryo quality, *PR* pregnancy rate, *LBR* live birth rate

not only the process of oocyte fertilization but also the development and the health of the offspring indicating that the role of sperm chromatin is more complex than previously believed. Hence, assessment of the sperm chromatin status may be of help to predict and improve the success of ARTs.

Currently, there are few tests available to evaluate sperm chromatin status, which have been used to study the involvement of such sperm feature in fertilization and embryo development in ARTs.

In this chapter, we illustrate the sperm chromatin structure and organization. Additionally, we describe the sperm chromatin abnormalities frequently found in human mature spermatozoa. We also review the evidences reported in literature regarding the association between sperm chromatin abnormalities and male infertility as well as natural and assisted reproduction outcomes.

Finally, we describe the actual tests used in research laboratories to detect sperm chromatin anomalies in order to understand their potential employment in clinical practice for the diagnosis of male reproductive health.

Chromatin Structure and Organization

In somatic cells, chromatin is constituted by proteins, DNA, and RNA that are assembled in a highly compacted form to allow the packaging of the genome in the cell nucleus [2]. In humans, the length of these molecules exceeds considerably the diameter of the nucleus; however, they are organized in repeated units called nucleosomes leading to a tight compaction. The nucleosome consists of two super helixes of DNA wrapped around a core of eight histones formed by two copies of H2A, H2B, H3, and H4 [3]. Nucleosomes are joined to each other by "linker DNA," of species-specific length, which is bound by the lysine-rich linker histone H1, helping chromatin to fold into higher-order structures. These nucleosomes are condensed in solenoids that in turn are further compacted in loop domains [4]. Although DNA packaging in somatic cells is an efficient process, it is not sufficient for spermatozoa which need to protect paternal genome during the transit in male and female genital tracts. In order to deliver a paternal "safe" genome to the oocyte, spermatozoa compact their DNA by about tenfold with respect to a somatic cell (Fig. 3.1). Such level of compaction is reached during spermatogenesis by the replacement of most of sperm histones with arginine- and cysteine-rich proteins called protamines [5] (Fig. 3.2). Protamine transcription occurs in round spermatids when meiosis is completed, but translation is repressed for several days through the binding of RNA repressor proteins to 3'-UTR or to a poly(A) tail. In elongated spermatids, repressors are removed, and the synthesis of protamines can start [6]. All vertebrates express protamine 1 (P1), whereas only some mammalian species (such as men and mouse) express also protamine 2 (P2, [7]). Chromatin remodeling is a gradual process that begins with the incorporation of histone variants, and then, histone hyperacetylation occurs facilitating nucleosome disassembly and the replacement of the histones, at first, by transition nuclear proteins (TPs) and, finally, by protamines [8].

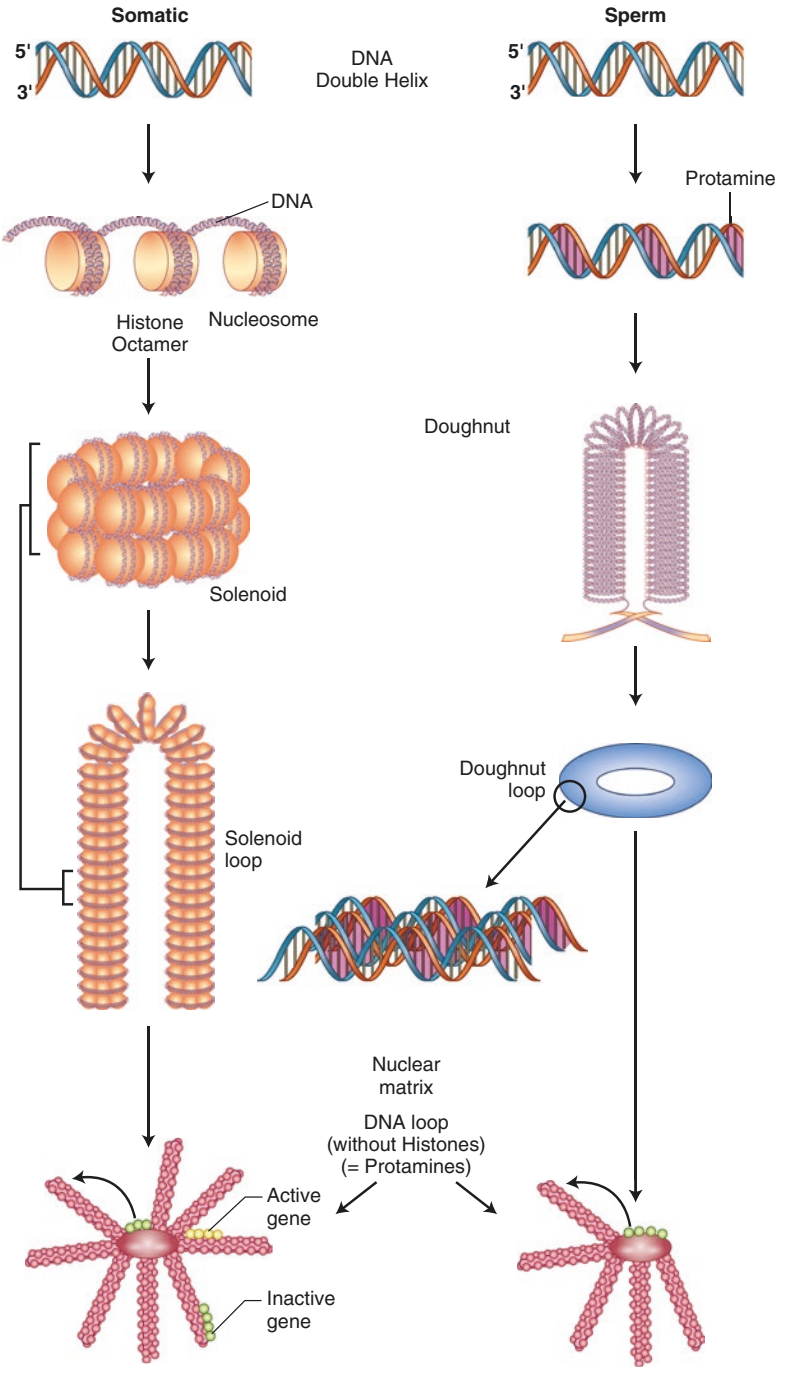

Fig. 3.1 Schematic representation of DNA structure in somatic and sperm cells. In somatic cells, DNA is organized into repeated units called nucleosomes. Such complexes are then condensed in solenoids that in turn are further compacted in loop domains. In spermatozoa, the histones are replaced by protamines. Protamine-bound DNA is coiled into doughnut in order to reach a tightly packed structure. Each doughnut represents a loop domain which is attached to the sperm nuclear matrix [4]

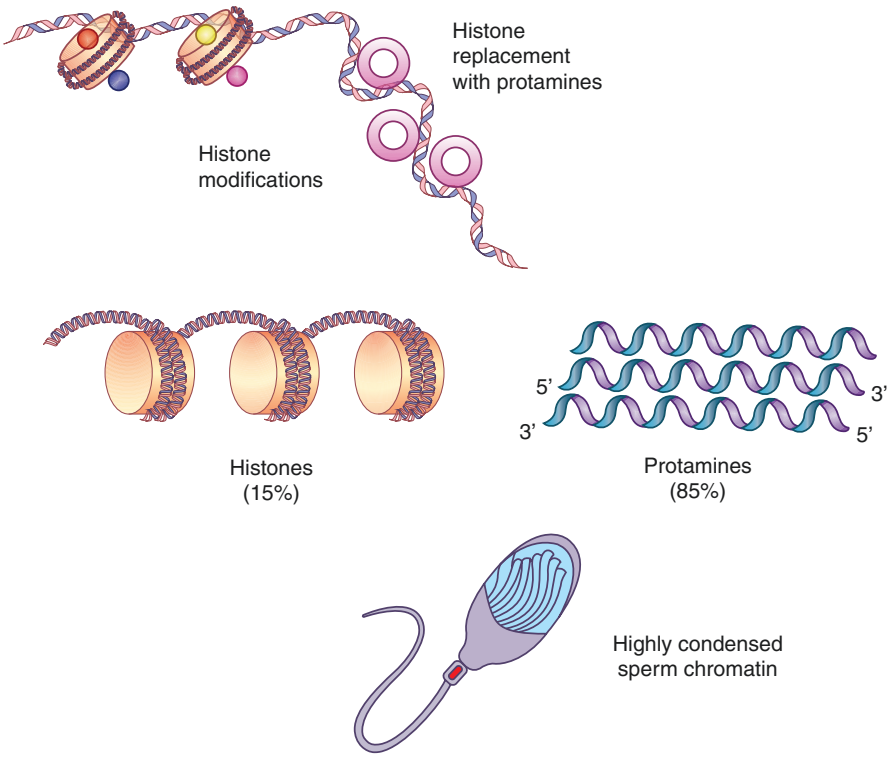

Fig. 3.2 Histone replacement by protamines in human spermatozoa. During spermatogenesis, most of sperm histones are replaced by arginine- and cysteine-rich proteins called protamines. This replacement is facilitated by post-translational histone modifications. Mature spermatozoa retain normally a percentage of histones different by species. In humans, histone retention does not exceed 15%. Chromatin remodeling allows obtaining a highly condensed sperm structure which protects paternal genome

Histone variants are differently expressed both temporally and spatially during germ cell development to facilitate histone to protamine transition [1]. As an example, testis-specific histone variant TH2B replaces canonical H2B histone during meiotic prophase destabilizing nucleosomes [9]. Moreover, histones undergo post-translational modifications (such as ubiquitination, acetylation, methylation, and phosphorylation) of the amino-terminal end which are involved in modulation of their activity. In particular, H2A, H2B, and H4 histones are acetylated in pre-meiotic cells (spermatogonia and primary spermatocytes) and under-acetylated during meiosis (secondary spermatocytes) and in most round spermatids. Histones become hyperacetylated in the last phases of spermiogenesis (elongating spermatids) likely to favor chromatin condensation [10].

TPs are arginine- and lysine-rich proteins exclusively localized to the nuclei of elongating and condensing spermatids. The principal TPs are TP1 and TP2, both encoded by single copy genes Tnp1 and Tnp2, respectively. Tnp1- or Tnp2-null

mice [11] are fertile likely because a compensation mechanism is activated. Conversely, mice with the double knockout of Tnp1 and Tnp2 are sterile and, despite a normal nuclear protamine deposition, are characterized by an altered chromatin condensation and presence of DNA breaks in spermatids [11, 12]. KO animal studies reveal that protamines (both P1 and P2) are essential to produce structurally and functionally intact sperm in mice [13, 14]. P1 consists of 49–50 amino acids and contains a central arginine-rich DNA-binding domain flanked on both sides by a short peptide containing cysteine residues. P2 has 50–70% (depending on the species) sequence identity with P1; however, it is slightly larger and has a higher histidine and lysine content. P2 is synthesized as a precursor of 103 amino acids that undergoes proteolytic processing after binding to DNA [15]. In humans, the ratio P1/P2 is approximately 0.9–1.0, and it is strictly regulated being critical for fertility status [16]. Both the P1 and P2 proteins are highly basic, a characteristic that leads to a strong intermolecular attraction with the negative charge of DNA backbone. After binding, the polyanionic DNA is transformed into a neutral polymer where adjacent DNA molecules are packed close together. This structure is further stabilized, during epididymal transit, by formation of disulfide bridges among the numerous residues of cysteine of protamines. At the end, sperm chromatin is folded into toroids consisting of a packed 50 kb DNA loop. Toroids are connected to each other by DNA strands which are more sensitive to nuclease action than DNA contained in protamine-bound toroids (Fig. 3.1). Such DNA strands, called "toroid linker regions," are the sites of attachment of DNA to nuclear matrix in the so-called matrix attachment regions (MARs) [5]. In mature spermatozoa, approximately 85% of the DNA is associated with protamines, whereas 15% remains associated with histones or other proteins [17] (Fig. 3.2). It has been demonstrated by Wykes and Krawetz [18] that histones and protamines are not randomly distributed in the sperm nucleus but they are located in certain repetitive sequences. In particular, Wykes and Krawetz [18] reported that histones are mostly localized in MARs, becoming more vulnerable to the action of nucleases. Histones and protamines have specific and different functions during reproduction process. Indeed, protamines are devoted to protect paternal genome as demonstrated also by the resistance of sperm DNA to mechanical insults [19]. On the other hand, the fact that round spermatids (lacking of protamines) are able to originate normal puppies if injected into the oocyte suggests that protamines are not required for embryogenesis [20]. Conversely, regions rich in histones seem to contain genes important for early embryo development after fertilization [21, 22]. However, these results have not been confirmed by other authors, who reported that the majority of putative nucleosome-binding sites are located in distal intergenic and intronic regions [23]. Despite lack of consensus on their location in sperm chromatin, histones seem to have a clear role in the control of embryonic gene expression by carrying various post-translational modifications, which represent an epigenetic code capable of influencing the development of embryo [1]. Such epigenetic signatures are transferred to the oocyte at fertilization allowing access of transcription factors to DNA to regulate gene expression during early embryo development [24].

Overall, the epigenetic inheritance is transferred to the oocyte through four different molecular mechanisms: complex organization of chromatin and its components and DNA methylation of the sperm genome and non-coding RNA [1].

The prenatal phase of germ cell development is characterized by complete demethylation of DNA, whereas in the postnatal phase, CpG methylation increases [25].

During spermatogenesis, different types of small RNAs are present in male germ cells. For example, pachytene spermatocytes and early spermatids are rich in miRNAs, playing a possible role in post-transcriptional silencing of genes during spermatogenesis to allow transcription at the right moment [26].

Imprinting errors may induce disorders in embryo development as well as in the offspring [27, 28]. It has been demonstrated that toxicant exposure, some nutrients, stress, and smoking may contribute to sperm epigenetic modifications that are transmitted transgenerationally, creating altered phenotypes [29].

Origin and Mechanisms of Chromatin Damage

As mentioned, a normal sperm chromatin structure and an intact DNA are important for the success of fertilization and for a correct embryo development. Several clinical studies have demonstrated that infertile men have substantially higher sperm DNA damage and an abnormal chromatin structure compared to fertile men [16, 30, 31]. Wrong chromatin packaging during spermatogenesis may be due to alterations of the molecular mechanisms involved in the process or to mutations in protamine and histone genes. In addition, exogenous factors can contribute to alter sperm chromatin/DNA structure such as male urogenital tract infections or exposure to toxic agent. In humans, a correct chromatin structure is guaranteed by the presence of equal quantities of P1 and P2, as an alteration of P1/P2 ratio has been associated with infertility [32]. Increased P1/P2 ratio could result from an abnormal processing of the P2 precursor [33] or from failure of replacement of histones by protamines [34]. Indeed, higher levels of P2 precursors [33] and histones [35] are found in infertile compared to fertile men. Another possible mechanism responsible for an altered P1/P2 ratio is the occurrence of mutations in genes encoding for protamines (PRM1 and PRM2). Several groups identified different SNPs in both PRM1 and PRM2 that may be associated with poor semen quality. The most frequent variant found in PRM1 is a change of one arginine with a serine residue in position 34, even if its pathogenicity has not been definitely proven [36].

Protamine deregulation may occur at multiple steps, including transcription. Aoki et al. [37] observed that elevated protamine mRNA retention is associated with aberrant protamine expression in infertile men. The increase of mRNA content is probably due to a defect in the regulation of protamine translation [37]. Also a proper phosphorylation of P2 is needed for replacement of TP2. Indeed, it has been described that knockout mice for Camk4 (the gene encoding for the protein responsible for P2 phosphorylation before its cleavage) are infertile due to the retention of

TP2 and absence of P2 [38]. Altered protamine ratio, therefore, seems to reflect deficiency or abnormal function of these accessory proteins leading to incomplete protein processing and unsuccessful DNA binding.

Protamine deficiency leads to a reduction of inter- and intramolecular disulfide bonds which are also necessary for chromatin condensation and stability, increasing the susceptibility of spermatozoon to DNA damage. It has been reported that men with reduced protamine content or abnormal P1/P2 ratio [39, 40] exhibit increased DNA fragmentation, characterized by single or double-stranded DNA breaks. In addition, sorted DNA-fragmented spermatozoa demonstrated higher histone persistence [41, 42]. It should be also considered that to facilitate histone to protamine transition, temporary nicks are introduced by topoisomerase II to relax DNA. Although such DNA breaks are next re-ligated, if alterations in the repairing system occur, spermatozoa with DNA fragmentation may reach the ejaculate [43]. A systematic review reports that protamine deficiency is significantly associated with sperm DNA fragmentation [44].

However, alterations in sperm chromatin packaging are not the only cause of sperm DNA fragmentation, as also abortive apoptosis, and oxidative stress have been reported to cause damage to DNA [42]. The concept of abortive apoptosis derives from evidence of presence of spermatozoa with apoptotic signs as well as DNA breaks in the ejaculate indicating that defective spermatozoa fail to complete the process of programmed cell death [45]. When ROS are produced in excess with respect to antioxidants, they may have pathogenic effects on spermatozoa, among them producing DNA brakes and base oxidation [46].

It is well known that several lifestyle factors including weight, smoking, diet, exercise, psychological stress, caffeine consumption, alcohol consumption, and exposure to environmental pollutants impact semen quality altering sperm morphology and motility and also the chromatin organization and DNA integrity. Studies on animal models demonstrated that alcohol abuse compromises both sperm chromatin maturity and DNA integrity [47, 48]. In humans, it is reported that cigarette smoking affects protamine levels with an increase in P1/P2 ratio [49]. Furthermore a study of Yu and colleagues [50] suggests that smoking may interfere with the transcription of protamine mRNA, leading to an abnormal sperm histone replacement.

Human sperm chromatin is susceptible not only to environmental factors but also to pathophysiologic conditions such as male urogenital tract infections, which are considered an important cause of male infertility. Some bacteria such as *Escherichia coli*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, and *Chlamydia trachomatis* can interact with spermatozoa damaging its DNA [51]. A study, conducted on semen samples infected with different bacteria, reports sperm protamine deficiency in infected patients [52].

Moreover, cancer itself and the subsequent therapeutic treatments to cure it may affect male fertility also by altering sperm DNA. O'Flaherty et al. [53] compared the quality of sperm DNA from patients with Hodgkin's lymphoma or testicular cancer to healthy volunteers, demonstrating that spermatozoa of patients show higher rates of DNA damage and decreased chromatin compaction compared to healthy men. The same authors, some years later, showed that the chemotherapy

provokes significant sperm DNA damage, compromising its structure and integrity up to 2 years after treatment [54]. Sperm cryopreservation, a procedure extensively used in *in vitro* fertilization protocols, is another cause of sperm DNA damage. After freeze-thawing, the integrity of sperm DNA worsen especially in those subjects with poor semen quality that probably have less chromatin condensation and therefore are less resistant to damage from cryopreservation [55, 56].

Sperm Chromatin and Male Infertility

Aberrant protamine expression or structure negatively affects the male fertility status. Animal studies demonstrated that deletion of one allele of P1 results in spermatozoa with decondensed chromatin, abnormal morphology, reduced motility, and enhanced acrosome reaction [57]. PRM2$^{-/-}$ mice are not able to reproduce [58]. Human studies suggest that the ratio P1/P2 is more crucial for fertility than the amount of the individual P1 and P2. Indeed, numerous studies reported a greater incidence of abnormal ratio among subfertile subjects with respect to controls [59–61]. Also, a recent meta-analysis demonstrated that both protamine mRNA and P1/P2 ratio are higher in subfertile men compared with controls [44]. In 87% of cases, the increase of protamine ratio is due to an under-expression of P2 and, only in 13% of cases, to an overexpression of P1 [44]. In addition, men exhibiting aberrant P1/P2 ratio likely display a decrease in sperm concentration, motility, and normal morphology [16, 39]. As mentioned above, besides condensing DNA to protect it, protamines have also the role of transmitting the epigenetic information necessary for the reactivation of the paternal genome after fertilization. Consequently, an abnormal protamine expression may have negative effects on the embryo development. Studies conducted on semen samples of male partners of couples undergoing ART cycles are very useful to clarify this issue, as it is possible to trace the entire process from fertilization to embryo development. A study including 338 subjects (32 normozoospermic volunteers and 306 patients undergoing ART) reveals that sperm protamine mRNA ratio is significantly higher in the control group compared to the ICSI group and men with normal mRNA ratio have a higher fertilization capacity in both IVF and ICSI cycles [62]. Similar results are also found when the protein expression of protamines is investigated: an aberrant P1/P2 ratio is associated with low fertilization rate and poor embryo quality [40]. Less consistent results are found regarding the relationship between P1/P2 ratio and pregnancy rate [40, 63]. Overall, these results suggest that P1/P2 ratio represents a good prognostic marker that could be used in addition to standard semen analysis in the diagnostic work-up of male infertility.

The balance between P1 and P2 can be compromised not only by an abnormal expression of protamines but also by an aberrant replacement of histones. A greater persistence of histones represents chromatin immaturity that can negatively affect *in vitro* fertilization. Most studies reported that chromatin immaturity is negatively related to fertilization and achievement of pregnancy [64–68]. Simon et al. [69] did

not find any association between histone retention and early ART outcomes, although a significant correlation was found with embryo development on Day 2 and Day 3. To note, most of these studies did not consider female factors that also influence ART outcomes. In an adjusted model for confounding factors including female age and female factors, sperm chromatin status has been demonstrated to predict the development of good-quality embryos with an odd ratio of 6.6 [68].

As described above, abnormal protamine content or aberrant histone replacement may also affect DNA stability making the DNA more susceptible to damage [40, 70]. Several meta-analyses explored the association between sperm DNA fragmentation and reproductive outcomes both in natural and in assisted reproduction. Results show that DNA damage is associated with a lower pregnancy rate in natural and assisted reproduction [71–73]. Live birth rate after IVF is higher in men with low DNA fragmentation levels [74], whereas miscarriage rate increases in subjects with elevated DNA damage [75]. Less clear is the relationship between DNA damage and ICSI reproductive outcomes, although a recent meta-analysis reports a negative impact on clinical pregnancy also after ICSI [76].

Assays to Evaluate Sperm Chromatin Status

It is clear from the studies described above that sperm chromatin structure impacts the outcome of both *in vivo* and *in vitro* fertilization, making it a possible marker useful in male infertility work-up. For this reason, in recent years, the techniques for evaluating the quality of sperm chromatin have gained increased value. Several assays have been proposed to assess sperm chromatin structure and packaging. Some methods are simple, easy to perform, and inexpensive and do not require sophisticated instrumentation allowing their use in virtually all clinical laboratories. Among these tests, those based on staining methods, with chromomycin A3 (CMA3), aniline blue (AB), and toluidine blue (TB), are most used. CMA3 is a guanine-cytosine-specific fluorochrome that competes with protamines for binding to DNA minor groove. CMA3 stains sperm DNA deficient of protamines, and therefore it is an indicator of poor DNA packaging [41]. Another approach used to evaluate sperm chromatin maturity is the AB test. This is a very simple and rapid methodology consisting in the staining of fixed samples with AB which is able to bind lysine residues of histones and therefore measures histone retention. Highly stained spermatozoa, displaying a dark-blue head, indirectly indicate the presence of a lower amount of protamines [41]. Unlike the CMA3 test, which requires a fluorescence microscope, the AB staining can be detected under an optical microscope. Both techniques may be employed as an index of a sperm immature nucleus. It should be noted that these tests are objective and operator-dependent and thus susceptible to interobserver variability and inter-laboratory variations.

Toluidine blue staining is a reliable test used to discriminate spermatozoa with abnormal chromatin structure (TB positive) from spermatozoa with normal chromatin packaging (TB negative). TB binds to phosphate groups of DNA strands when

chromatin proteins are more loosely electrostatically bound to the DNA, whereas a highly packaged chromatin is not accessible to the dye. For this reason, TB staining is used also as an indirect measure of sperm DNA integrity [77].

Other, more laborious and less popular, methods used to evaluate sperm chromatin structural elements foresee the measurement of nuclear proteins linked to DNA. According to these methods, nuclear proteins are extracted from semen samples by the use of decondensation buffer, and then proteins are separated by gel electrophoresis according to their molecular weight. Gel are stained and scanned to measure the intensity of bands related to protamines [21]. Other authors [49] evaluated protamine bands by Western blot using specific antibodies. In both cases, P1 and P2 concentrations are calculated from the standard curve generated from the human protamine standard included in each gel. Among human histone isoforms, the predominant H2B variant is usually evaluated. Results are expressed as P1/P2 ratio or H2B-to-protamine ratio [49, 78]. Some authors also report PRM1/PRM2 ratio performing a quantitative analysis of cDNA by real-time PCR [79].

Table 3.1 summarizes the data present in literature regarding the association between sperm chromatin status detected by the above described tests and ART outcomes. As can be observed, most studies report an association with fertilization rate (FR) or embryo quality (EQ). However, discrepancies exist depending on the assay used to evaluate chromatin maturation.

Conclusions

Over the past decade, there has been a growing body of research investigating the role of sperm chromatin in male factor infertility. Sperm chromatin with its highly compacted structure fulfills several key functions including modifications of the sperm nucleus in an ergonomic and hydrodynamic shape necessary to surmount the barriers to reach and fertilize the oocyte, protection of sperm DNA from physical and chemical insults, deprogramming, and deactivating the paternal genome prior to fertilization [80, 81]. These functions that are unique of sperm cells are allowed by the transition from histones to protamines during sperm maturation. The improper histone replacement or a deficient protamination may not only be a marker of abnormal spermiogenesis but also affect oocyte fertilization and reproductive outcomes. Several tests have been developed to determine chromatin maturity status that may be useful in male infertility work-up to add further information to standard semen analysis. In particular, the use of these techniques could be of help in cases of idiopathic infertility where traditional analysis does not find alterations in semen quality and no evident female reproductive system pathologies are diagnosed. In these cases, couples often refer to assisted reproduction programs where natural barriers are bypassed and a spermatozoon with abnormal chromatin could fertilize the oocyte. In view of the role of paternal histones in embryo development, an abnormal chromatin structure could lead to increased risk of epimutations to the offspring. Numerous studies have demonstrated an association between protamine deficiency

and fertilization rate, embryo quality, as well as achievement of clinical pregnancy. However, the usefulness of current methods to detect sperm chromatin status in clinical practice is still debated, partly for their drawbacks [82], and further research is needed in order to develop more specific and sensitive tests.

Finally, recent insights suggest that the male gamete must no longer be considered a silent vehicle of paternal genome to the oocyte as it plays a crucial role for epigenetic reprogramming of the zygote following fertilization in an orchestrated and sequential manner. Such new findings evidence the need to increase the knowledge about the complex nature of human sperm chromatin structure not only to answer the questions of basic cell biology but also to orient clinicians in the management of infertile men.

Review Criteria

A systematic review of the literature concerning sperm chromatin and reproduction was conducted. The following subject headings and keywords were searched:

(#1) "sperm chromatin structure" OR "sperm chromatin integrity" OR "sperm chromatin maturity" OR "sperm chromatin status" OR "sperm protamination" OR "sperm histone retention".
(#2) "spermatogenesis" AND (#1)
(#3) "male infertility" AND (#1)
(#4) "assisted reproduction" AND (#1)

Original research articles and reviews published in PubMed database in the last 25 years were considered, and those relevant for our chapter were selected. We also recorded the main assays used to evaluate sperm chromatin compaction, and we summarized the data regarding the association between sperm chromatin status detected by these tests and assisted reproduction outcomes.

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Chapter 4
Genetic Basis of Endocrine Regulation of Spermatogenesis

Julie W. Cheng and Edmund Y. Ko

Abbreviations

ARKO	Androgen receptor knockout
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
ERKO	Estrogen receptor knockout
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HPG	Hypogonadal-pituitary-gonadal
LH	Luteinizing hormone
SCARKO	Sertoli cell androgen receptor knockout

Key Points

- The hypothalamic-pituitary-gonadal axis promotes testosterone production and spermatogenesis through luteinizing hormone and follicle-stimulating hormone and can be impaired by disorders such as Kallmann syndrome or congenital hypopituitarism.
- Testosterone production occurs through a series of biochemical reactions. Deficiencies in the receptor for luteinizing hormone or enzymes involved in testosterone production can result in impaired spermatogenesis through hypovirilization.

J. W. Cheng (✉) · E. Y. Ko
Department of Urology, Loma Linda University Health, Loma Linda, CA, USA

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- High testosterone levels within the germinal epithelium and functional androgen receptors in Sertoli cells are necessary for spermatogenesis. Disorders of androgen receptors result in androgen insensitivity and subsequently affect spermatogenesis.
- Estrogen contributes to spermatogenesis by acting as a survival factor for germ cells, regulating fluid dynamics within the male reproductive system, and contributing to feedback inhibition of the hypothalamic-pituitary-gonadal axis.
- The hypothalamic-pituitary-gonadal axis is regulated by negative feedback through testosterone, estrogen, and inhibin. Androgen excess from exogenous hormones or congenital adrenal hyperplasia can cause endocrine dysfunction within this system.

Introduction

Spermatogenesis requires a well-coordinated interaction between the endocrine system and testicular parenchyma. The hypothalamic-pituitary-gonadal (HPG) axis contributes to both the activation and regulation of this process, and endocrine dysfunction can subsequently impair spermatogenesis. In addition to acquired causes, genetic disorders can impair coordination between hormone production, hormone release, and receptor function to ultimately limit spermatogenesis. The purpose of this chapter is to describe hormonal regulation of spermatogenesis with consideration for the genetic basis of this regulation. Genetic disorders affecting endocrine regulation of spermatogenesis will be discussed as well.

The Hypothalamic-Pituitary-Gonadal Axis

The HPG axis (Fig. 4.1) is a component of the endocrine system that drives sexual development and reproduction. The hypothalamus is located at the base of the cerebrum and responds to diurnal patterns and environmental conditions. To regulate homeostasis through positive and negative hormonal feedback loops [1], the hypothalamus provides direct neural signaling to the posterior pituitary and hormonal signaling to the anterior pituitary via the hypophyseal portal system. Gonadotropin-releasing hormone (GnRH) is released by the hypothalamus in pulsatile rhythmic secretions [1–3] and acts upon the anterior pituitary.

The anterior pituitary is located within the sella turcica at the base of the cranium adjacent to the hypothalamus. Among cells responsible for the production of several fundamental hormones, gonadotrophs contain GnRH receptors that are coded by the *GNRHR* and *GNRHR2* genes that are respectively located at chromosomes

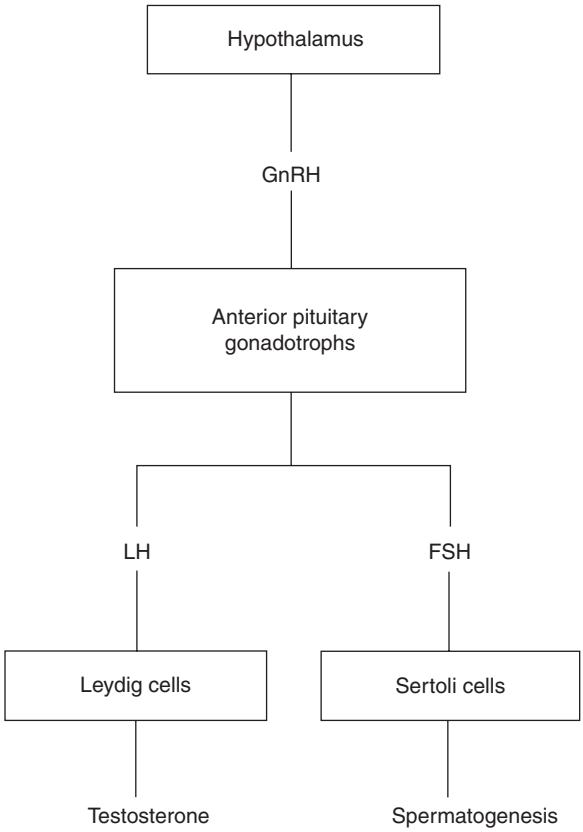

Fig. 4.1 The hypothalamic-pituitary-gonadal axis

8p11.2-p21 and 20p13 [4]. Gonadotrophs respond to GnRH by producing luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones are released into the pituitary efferent vein to enter systemic circulation and act upon the testis. As glycoprotein polypeptide hormones, LH and FSH act on different components of the testis by binding to G protein-coupled receptors that activate adenylate cyclase and increase intracellular concentrations of cyclic AMP. Luteinizing hormone acts upon Leydig cells to promote testosterone production, and FSH acts upon Sertoli cells to promote spermatogenesis.

Disorders of the Hypothalamic-Pituitary-Gonadal Axis

When the production or activity of GnRH is impaired, there is a downstream reduction of LH and FSH activity that results in hypogonadotropic hypogonadism. This occurs in patients with Kallmann syndrome, congenital hypogonadotropic hypogonadism, and idiopathic hypogonadotropic hypogonadism. Multiple genetic defects have been linked to Kallmann syndrome, which can have autosomal

dominant, autosomal recessive, and X-linked patterns of inheritance [5]. *KAL1* is an X-linked gene located at chromosome Xp22.3 that is among the more commonly identified genes linked to Kallmann syndrome [6]. As a result of GnRH failure, patients with hypogonadotropic hypogonadism exhibit delayed puberty, poorly defined sexual characteristics, and azoospermia [5]. Anosmia is unique to patients with Kallmann syndrome, and olfactory bulb anomalies can be radiologically demonstrated in these patients [7].

Hypogonadotropic hypogonadism can also occur when gonadotropes in the anterior pituitary fail to respond to GnRH signaling. Congenital hypopituitarism is a rare disorder that arises from disorders of the PIT-1 transcription factor on chromosome 3p [8]. Mutations impair differentiation of the anterior pituitary gland with subsequent functional impairment [9]. Patients exhibit a short stature due to lack of pituitary hormones, including growth hormone, thyroid-stimulating hormone, and prolactin [9]. While somatotropes, thyrotropes, and lactotropes are primarily affected, gonadotropin deficiency has been described with low testosterone and impaired spermatogenesis [10].

Androgen Production

Luteinizing hormone activates Leydig cells and stimulates testosterone production through LH receptors that are coded by the *LHCGR* gene located on chromosome 2p21 [11]. Stimulation from LH mobilizes cholesterol into the mitochondria where it is converted into pregnenolone. Pregnenolone is then transported to the smooth endoplasmic reticulum and undergoes a series of reactions to become 17α-hydroxypregnenolone, dehydroepiandrosterone (DHEA), androstenedione, and finally testosterone (Fig. 4.2). As a steroid hormone, testosterone is able to diffuse directly through the cellular membrane.

Testosterone diffuses out of Leydig cells into local capillaries to enter systemic circulation. This hormone is reversibly bound by sex hormone-binding globulin and albumin for transport. When released by these circulating proteins, testosterone diffuses directly into cells to bind androgen receptors within nuclei and initiate transcription for protein synthesis. While testosterone serves as the primary male sex hormone that contributes to sexual development and characteristics, it can also be converted to dihydrotestosterone (DHT) by 5α-reductase, which is coded by the *SRD5A2* gene located at chromosome 2p23 [12]. Both testosterone and DHT provide male hormonal influence throughout embryonic development, pubertal growth and maturation, and adulthood. Despite the multiple systemic effects of testosterone, the significant contribution of testosterone to spermatogenesis occurs through paracrine signaling on adjacent tissues within the germinal epithelium and testicular interstitium.

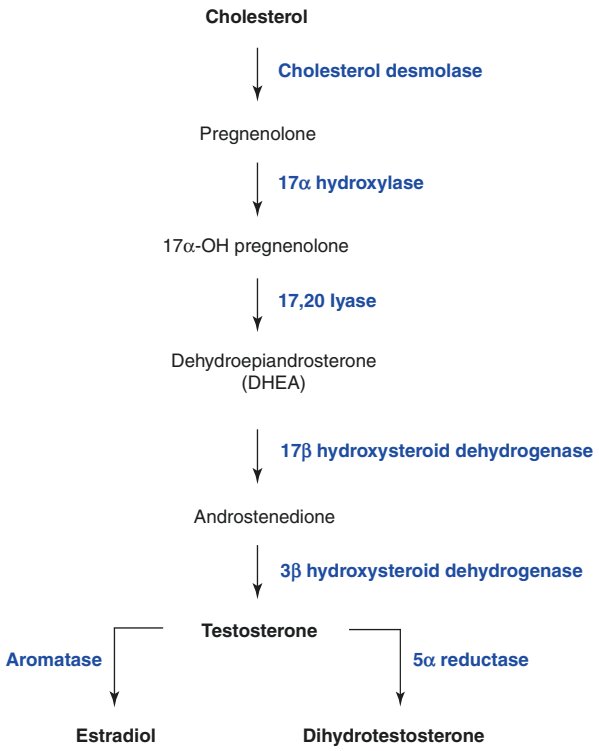

Fig. 4.2 Biosynthetic pathway of testosterone from cholesterol

Disorders of Androgen Production

Aberrations or deficiencies in the LH receptor or any of the enzymes involved in androgen production (Fig. 4.2) can limit the production of testosterone and subsequently result in hypovirilization. Although it is rare, isolated LH deficiency has been described in patients with idiopathic mutations of the LH β unit limiting the function of LH and resulting in low testosterone levels and a eunuchoid body habitus [13, 14]. Testosterone insufficiency can also result from enzymatic defects in testosterone production. As multiple mutations have been identified for these enzymes [15–19], phenotypic expression and hypovirilization can be variable and depend on the degree of the enzymatic defect and subsequent testosterone deficiency. 5α-Reductase deficiency limits the conversion of testosterone to DHT. Low plasma DHT levels can cause incomplete masculinization of the external genitalia, and patients subsequently have internal male reproductive tracts with feminized external genitalia [20]. Although patients with 5α-reductase deficiency are raised female, testicular biopsy of patients with this condition nevertheless demonstrates impaired spermatogenesis with irregular sperm production [21].

Hormonal Influence Within the Germinal Epithelium

The germinal epithelium is comprised of Sertoli cells and germ cells that surround the central lumen of the seminiferous tubules (Fig. 4.3). Sertoli cells are columnar-shaped cells that are attached to the basement membrane of seminiferous tubules. Tight junctions between Sertoli cells create the blood-testis barrier, divide the adluminal and basal compartments of the seminiferous tubules, and anchor germ cells to the basement membrane to maintain the germ line. Developing sperm cells are sandwiched between Sertoli cells as they undergo proliferation, spermatogenesis, spermiogenesis, and apoptosis. Sertoli cells support spermatogenesis through the effects of both FSH and testosterone.

Follicle-stimulating hormone binds receptors in Sertoli cells to promote cell growth, protein production, and spermatogenesis [22]. The human *FSHR* gene for the FSH receptor has been localized to chromosome 2p21-p16 [23, 24]. Although FSH receptor knockout has been shown to inhibit folliculogenesis and result in infertility in the female mouse [25, 26], this does not cause infertility in male mice [25–27]. These male mice, however, exhibit a reduction in testicular size [25, 26] and poorer sperm quality [28]. Furthermore, FSH receptor mutations can similarly impair fertility in female patients but do not completely impair fertility in men [29, 30]. Similar to LH deficiency, isolated FSH deficiency has also been described in men with mutations of the FSH β unit, normal virilization, and testosterone levels but poor sperm count and motility as a result of low FSH levels [31–33]. While FSH primarily optimizes spermatogenesis and germ cell count, it may not be necessary for male fertility. Instead, the contribution of FSH to spermatogenesis may be indirect by stimulating Sertoli cells to express androgen-binding protein and androgen

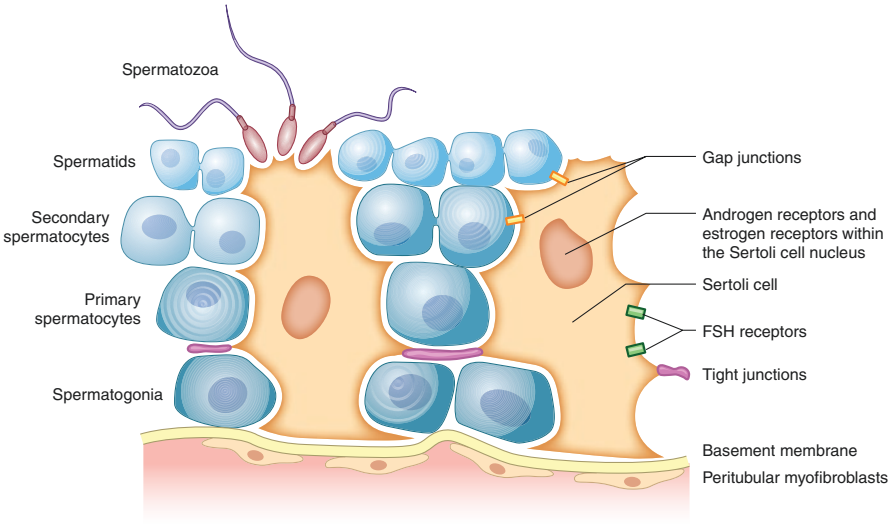

Fig. 4.3 The germinal epithelium comprised of Sertoli cells and germ cells

receptors, sequester testosterone to the germinal epithelium, and subsequently create a testicular microenvironment that promotes germ cell development [34].

The testicular microenvironment contains a high level of testosterone. Androgen-binding protein, which is produced by Sertoli cells, sequesters testosterone to maintain intratesticular testosterone levels that are approximately 40-fold greater than serum levels [35]. These high levels of testosterone are necessary for spermatogenesis [35, 36]. In fact, suppression of intratesticular testosterone levels to serum levels can result in a significant decline in sperm count by up to 98% [35]. In contrast, supplementation with testosterone and DHT can act upon the androgen receptor and restore spermatogenesis even when FSH is absent in hypogonadal mice genetically deficient in GnRH [37].

The human androgen receptor is a nuclear receptor that is coded by the *AR* gene localized to the X chromosome Xq11–12 [38–40] and expressed in Sertoli cells, Leydig cells, and peritubular myoid cells [41, 42]. As germ cells do not express a functional androgen receptor [41], androgen regulation of spermatogenesis is primarily mediated by Sertoli cells [43] through gap junctions that exist between these cells with germ cells [44, 45].

Direct androgen stimulation to Sertoli cells is required for spermatogenesis [36, 42, 43, 46, 47]. While androgen receptor knockout (ARKO) mice with a complete androgen insensitivity phenotype are infertile due to small intra-abdominal gonads, aberrant embryologic development, and disrupted germ cell development [42], androgen receptor blockade at Sertoli cells only also results in infertility. Despite normal testicular descent, male reproductive tract development, Sertoli cell count, hormonal profile, and androgen receptors at peritubular myoid cells and interstitial tissue of the testis [36, 42], Sertoli cell androgen receptor knockout (SCARKO) mice exhibit meiotic arrest during spermatogenesis [36, 42]. There is a significant reduction in spermatocytes, round spermatids, and elongated spermatids with no production of elongated spermatids [42]. Furthermore, there is also an increased rate of germ cell apoptosis [42]. This suggests that direct androgen action on Sertoli cells is required for germ cell development and survival.

Loss of androgen stimulation to Sertoli cells also results in structural changes within the germinal epithelium and testicular microenvironment of SCARKO mice [42, 43, 47]. Stereological analysis demonstrates displacement of Sertoli cell nuclei as well as a reduction in seminiferous tubule diameter that would suggest dysfunctional fluid secretion [42, 43]. Furthermore, Leydig cell development and function is impaired in both ARKO and SCARKO mice [43, 47], and testis weight is reduced by 28% [43].

Androgen receptors have also been identified in peritubular myoid cells within the testis. While the influence of these specific receptors on spermatogenesis continues to be elucidated, these cells may provide an underappreciated contribution to male fertility. Select androgen receptor knockout at peritubular myoid cells resulted in an 86% reduction in germ cells, impaired Sertoli cell function, and infertility in male mice [48]. Despite the contribution of these cells to spermatogenesis, androgen stimulation of these cells alone without Sertoli cell stimulation does not appear to be sufficient for spermatogenesis [36].

Disorders of the Androgen Receptor

Androgen insensitivity can result from a loss of androgen receptor function despite testosterone production. Over 600 mutations of the androgen receptor gene have been identified [49]. This can manifest as either complete or partial androgen insensitivity syndromes. Patients with complete androgen insensitivity do not have testosterone stimulation during embryonic development and are subsequently phenotypically female with a 46XY karyotype and cryptorchid gonads [50, 51]. Partial androgen insensitivity, in contrast, can vary in the degree of responsiveness to androgens. Men with mild androgen insensitivity syndrome can present as undervirilized males with infertility and no genital anomalies [51, 52].

The Role of Estrogen in Spermatogenesis

Testosterone can be converted by aromatase to estrogen, which may also significantly contribute to spermatogenesis as aromatase and estrogen receptors have been localized to testicular parenchyma and germ cells [53, 54]. Disruption of either of these proteins can impair sperm production, motility, and function despite normal testosterone levels [55–57].

Aromatase is a component of the cytochrome P450 enzymes and is coded by the *cyp19* gene located on chromosome 15q21.1 [55, 58]. Aromatase knockout mice do not exhibit any changes to Sertoli cells, early germ cells, or hormone levels of FSH and androgens [55]. However, these mice ultimately develop progressive infertility as spermatogenesis becomes arrested at spermiogenic stages with a significant reduction in round and elongated spermatids [55]. This suggests that aromatase may be necessary for spermatogenesis through the direct actions of estrogen [55] and estrogen may be a survival factor for germ cells as 17β-estradiol inhibits apoptosis of spermatocytes and spermatids [54].

There are two forms of nuclear estrogen receptors that are activated by 17β-estradiol. Estrogen receptor α (ERα) is encoded by the *ESR1* gene localized to chromosome 6q25.1 [59], and estrogen receptor β (ERβ) is encoded by the *ESR2* gene localized to chromosome 14q22-24 [60]. Both estrogen receptors have been localized through immunohistochemical analysis and Western blot analysis to be in male reproductive tissues, including Sertoli cells, germ cells, Leydig cells, and the epididymis [41, 54, 60]. Estrogen receptor knockout (ERKO) mice have normal testes until puberty and do not initially exhibit anatomic and functional anomalies [56]. However, similar to aromatase knockout mice, ERKO mice later exhibit disrupted spermatogenesis through reduced sperm motility and function as these mice become increasingly infertile [56]. Estrogen may also regulate luminal fluid dynamics within the seminiferous tubules, epididymis, and efferent ductules for sperm transport as these structures become dilated in ERKO mice and degenerate later in life [56, 57, 61]. Although testosterone is elevated with normal LH and FSH levels in these mice, their testes become atrophic, and male mice exhibit decreased mating

frequency [56]. However, a separate study found that ERα may not be necessary for germ cell development or function [62], and further studies are needed to define the role of estrogen in spermatogenesis. In addition to its direct impact upon the germinal epithelium, estrogen can also influence spermatogenesis through regulation of the HPG axis.

Regulation of the Hypothalamic-Pituitary-Gonadal Axis

The HPG axis is regulated through feedback inhibition (Fig. 4.4). Testosterone produced by Leydig cells and inhibin-B produced by Sertoli cells provide negative feedback to the HPG axis to suppress gonadotropin release. Activin and inhibin are hormones produced by Sertoli cells that respectively act upon the HPG axis to promote or suppress FSH secretion at the anterior pituitary. While inhibin-B suppresses FSH secretion, testosterone provides negative feedback to both the hypothalamus and anterior pituitary to inhibit respective GnRH and LH secretion. Testosterone has been specifically demonstrated to alter the threshold and frequency of the neuronal firing pattern within the hypothalamus [63, 64]. The influence of testosterone on the HPG axis has been demonstrated by studies evaluating the reduction in intratesticular testosterone levels through feedback inhibition from anabolic steroid use, testosterone replacement therapy, and exogenous testosterone as a potential male contraceptive [65–68].

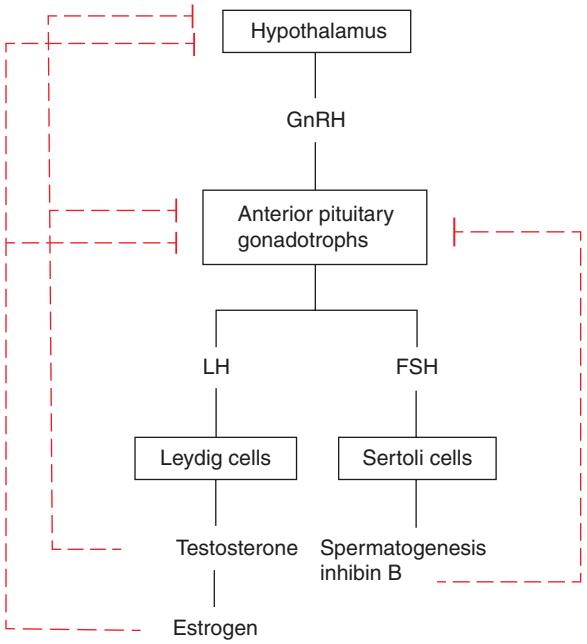

Fig. 4.4 Feedback inhibition of the hypothalamic-pituitary-gonadal axis

Testosterone also indirectly provides negative feedback to the hypothalamus and anterior pituitary in the form of estrogen. Similar to testosterone, estradiol also mediates negative feedback by affecting GnRH neuron activity patterns [64] and mRNA expression [69]. While castrated male mice demonstrated an increase in excitatory hyperpolarization currents within the GnRH neurons of the hypothalamus, estradiol replacement reversed the effects of castration by decreasing these currents and restoring the hypothalamic GnRH neuron firing patterns to baseline levels [63, 64]. Furthermore, aromatase knockout mice that lack estrogen production and its inhibitory effects exhibit increased LH levels with associated Leydig cell hyperplasia [55].

Disorders of Hypothalamic-Pituitary-Gonadal Axis Regulation

Testosterone production and spermatogenesis can be limited by conditions in which excess androgens cause feedback inhibition at the HPG axis. This can occur with increased androgen production by the adrenal gland. Among the various causes of congenital adrenal hyperplasia, 21-hydroxylase deficiency is the most common [70]. 21-Hydroxylase is located at the *cyp21* gene on chromosome 6p21.3 and contributes to the production of aldosterone and cortisol in the adrenal gland. Deficiency of this or other adrenal enzymes limits production of mineralocorticoids and glucocorticoids and, instead, shunts steroid production to androgens. This can result in infant salt-wasting syndromes, virilization in females, and precocious puberty in males. The associated increase in endogenous androgen causes negative feedback at the HPG axis. Males with congenital adrenal hyperplasia exhibit variable fertility, but oligospermia and azoospermia have been reported in these patients [71, 72].

Conclusion

There are various opportunities for endocrine dysfunction that can result in hypogonadotropic hypogonadism through a loss of circulating hormones or their respective receptors. Hormonal signaling from the hypothalamus activates gonadotropin release into systemic circulation before reaching the testicle. Testosterone is then produced from cholesterol through a series of biochemical reactions and must be sequestered by Sertoli cells within the germinal epithelium to maintain high intratesticular testosterone levels. Additional Sertoli cell contributions that establish a testicular microenvironment conducive to germ cell development also require a functional androgen receptor in addition to contributions from other steroidal hormones. This process is ultimately kept in balance through feedback inhibition of the HPG axis. Various genetic disorders (Table 4.1) can alter the ability of the endocrine system to activate and regulate spermatogenesis. As the knowledge of genetic function and disorders continues to grow, further studies will contribute to the understanding of the genetic basis of endocrine regulation of spermatogenesis.

Table 4.1 Endocrine disorders affecting spermatogenesis through hypogonadotropic hypogonadism

Condition	Gene (locus)	Fertility impairment	Clinical manifestations
Kallmann syndrome	*KAL1* (Xp22.3)	Deficient GnRH	Midline facial defects, anosmia, renal dysplasia
Congenital hypopituitarism	*PIT-1* (3p)	Deficient LH and FSH in rare cases	Hypopituitarism and short stature
Isolated LH deficiency	Unknown	LH β subunit	Eunuchoid body habitus
Isolated FSH deficiency	Unknown	FSH β subunit	Normal virilization and testosterone levels
FSH receptor mutation	*FSHR* (2p21-p16)	Impaired FSH signaling	Normal virilization and testosterone levels
Testosterone insufficiency	*cyp17* (10q24.3)	Impaired testosterone synthesis from enzymatic defects	Variable hypovirilization
5α-reductase deficiency	*SRD5A2* (2p23)	Lack of DHT conversion during embryonic development	Internal male reproductive tract with feminized external genitalia
Androgen insensitivity syndrome	*AR* (Xq11–12)	Androgen receptor	Complete: Phenotypically female with 46XY karyotype Partial: Variable hypovirilization
Congenital adrenal hyperplasia	*cyp21* (6p21.3)	Excess androgen production causes feedback inhibition of the HPG axis	Infant salt-wasting syndromes, virilization in females, precocious puberty in males

Review Criteria

- PubMed search for hypothalamic-pituitary-gonadal axis, endocrine dysfunction affecting spermatogenesis, and hypogonadotropic hypogonadism ($n = 122$):
  - Articles screened through title and abstract

    Excluded articles related to non-hormonal testicular dysfunction ($n = 38$)
    Excluded articles related to female reproduction ($n = 35$)
    Excluded articles related to medical management ($n = 12$)
  - There were 37 articles cited from the initial search
- Secondary PubMed search for specific localized genes for receptors and enzymes and specific disorders affecting endocrine regulation of spermatogenesis:
  - Articles screened through title and abstract ($n = 123$)

Excluded articles without specific gene and chromosome location ($n = 45$)
Excluded articles of other genetic disorders of spermatogenesis without endocrine dysfunction ($n = 43$)

  - There were 13 articles cited for specific genes for receptors and enzymes and 22 articles cited for specific disorders affecting endocrine regulation

- A total of 72 total articles were cited

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Chapter 5
Epidemiology of Genetic Disorders in Male Infertility

Marlon P. Martinez, Haitham Elbardisi, Ahmad Majzoub, and Mohamed Arafa

Key Points

- Despite exhaustive efforts to determine the exact nature of the causes of male infertility, many are still diagnosed with idiopathic male infertility.
- The true incidence and aetiologies of male infertility remain poorly studied and characterized.
- Geographical variation is believed to be the cause of different incidences of male infertility.
- The main genetic variants leading to male infertility are chromosomal alterations, inversions, translocation, Y chromosome microdeletions and gene mutations.
- A comprehensive male evaluation for all partners of infertile couples should be performed in order to uncover possible significant and treatable medical conditions before pursuing therapies with ART.
- Large-scale, prospective, epidemiological studies may enhance our understanding of the genetic disorders of male infertility.

M. P. Martinez
Philippine Urological Association, Department of Surgery, Section of Urology, University of Santo Tomas Hospital, Sampaloc, Manila, Philippines

H. Elbardisi · A. Majzoub · M. Arafa (✉)
Department of Urology, Hamad Medical Corporation, Doha, Qatar

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Introduction

Infertility, as defined by the recent consensus-based and evidence-driven set of terminologies set by the International Committee for Monitoring Assisted Reproductive Technologies (ICMART), is a "disease characterized by the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse or due to an impairment of a person's capacity to reproduce either as an individual or with his/her partner" [1]. Infertility affects 15% of couples desiring to have a child [2]. Male factor as a sole cause of infertility accounts for 20% of cases, while both male and female factors contribute in another 20–30% of cases [3]. Another study of male infertility data from five European countries showed that 6.4–42.4% of cases were due to male factors [4].

Aetiologies of male factor infertility are multifactorial. In 30–40% of cases, the cause of male infertility remains undiscovered, termed idiopathic [5]. Half of the cases of idiopathic infertility could be due to known or unknown genetic abnormalities [6]. The frequency of known genetic abnormalities increases with the severity of the spermatogenic defect. Numerical and structural defects comprise the main chromosomal abnormalities affecting paternity of men wanting to father a child [7]. This accounts for 6% of infertile men. Azoospermic men experience higher prevalence rates up to 15%.

With the advancement of assisted reproductive technologies (ART), such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), clinicians must know the role played by genetics in complex male factor infertility cases [8]. The frequency of the inheritance of mutations through these assisted reproduction procedures and their impact on future generations are not yet fully discerned.

Male factor infertility is a complicated disorder, wherein underlying aetiologies often remain undetected [5]. Future research on the genetics and molecular defects in sperm production and function is warranted to improve detection. This will lead to more focused treatment of men with genetic abnormalities. The prevalence of genetic anomalies warns clinicians of the importance of genetic testing in male factor infertility. This chapter will focus on the epidemiology of the common genetic disorders causing poor male reproductive potential, with clinical applications.

Epidemiology of Infertility

The true incidence and aetiologies of male infertility remain poorly studied and characterized [9]. In 2007, Boivin et al. performed an international estimate of infertility prevalence from 25 population surveys [10]. It was estimated that 72.4 million people were infertile. From this group, approximately 40.5 million people were seeking infertility medical care. Mascarenhas et al. estimated the prevalence of infertility between 1990 and 2010 in 190 countries [11]. The authors identified 277 demographic and reproductive health surveys and obtained the individual-level

questionnaire responses. In this systematic analysis of surveys, there was an increase in the absolute number of couples experiencing infertility, from 42.0 million in 1990 to 48.5 million in 2010. In America, an estimated seven million couples seek infertility care annually [12]. Data of 11,067 men from the National Survey of Family Growth performed by the Centers for Disease Control and Prevention to determine the frequency of male infertility evaluations showed that only 18% of couples did not complete a male infertility evaluation when the male partners were asked. This increased to 27% when 25,846 female partners were asked. This corresponds to 370,000–860,000 men who were not seen and examined during an infertility evaluation. In the latest result on the study by Agarwal et al., at least 30 million men were considered infertile [13]. The highest rates were observed in Africa and Eastern Europe. Across the world, Australia and Central and Eastern Europe had the most number of infertile men, consisting of 8–9% and 8–12%, respectively. Other regions, such as North America (4.5–6%), sub-Saharan Africa (2.5–4.8%) and Europe (7.5%), showed lower calculated percentages of infertile men.

A literature search of systematic reviews and meta-analyses of population-based studies was performed on the factors that affect male infertility [13]. Out of 86 relevant articles, 16 were included in the study. The percentage of male infertility cases globally varied from 2.5 to 12% (Table 5.1). The calculated global data revealed that 20–70% had a cause of infertility that was attributable to male factor [13]. This is a wide-range percentage compared to the commonly cited percentage of 20%, which was derived from a multicentre survey of 1686 couples in 3 French regions [3]. In this recent study by Agarwal, the calculated percentages of male infertility worldwide were based on a review of the current literature. This current study demonstrates the infertility cases in regions that were due to male factor (Table 5.2), which

Table 5.1 Global representation of infertile men

	Infertile men (%)
Sub-Saharan Africa	2.5–4.8%
North America	4.5–6%
Europe	7.5%
Australia	8–9%
Central/Eastern Europe	8–12%

Data from Agarwal et al. [13]

Table 5.2 Male factor involvement in different regions worldwide

	Male factor (%)
Asia	37%
Oceania	40%
Sub-Saharan Africa	20–40%
Africa	43%
North America	50%
Europe	50%
Latin America	52%
Central/Eastern Europe	55.73%
Middle East	60–70%

Data from Agarwal et al. [13]

included Asia (37%), sub-Saharan Africa (20–40%), Oceania (40%), Africa (43%), Europe (50%), North America (50%), Latin America (52%), Central/Eastern Europe (55.73%) and the Middle East (60–70%).

Geographical variation is believed to be the cause of different incidences of male infertility. Ikechebelu et al. evaluated 314 infertile couples from Southeastern Nigeria for the aetiologies of their poor reproductive potential [14]. Thirty-five percent (n = 110) of the cases had secondary infertility, while 65% (n = 204) had primary infertility. Male factor infertility was responsible for 42.4% of the infertile couples. The most common causes for male infertility were oligozoospermia and asthenozoospermia, amounting to 35.9% and 32.3%, respectively. In Western Siberia, 333 (16.7%) couples were considered infertile from 2000 randomly selected married couples in an epidemiological study conducted by Philippov et al. [15]. From the semen analysis of the men tested, 45.7% had identifiable abnormalities, while 54.3% remained idiopathic. Inflammatory disease of the male accessory gland was the most common cause of male infertility, amounting to 12.9% of cases, and 8.6% of these inflammatory cases resulted in obstructive azoospermia. Aflatoonian et al. studied the demographic characteristics of infertile couples in the province of Yazd, Iran [16]. Among 5200 couples, 5.52% (n = 277) experienced infertility. Of these infertile couples, 3.48% and 2.04% had primary and secondary infertility, respectively. The prevalence of infertility was higher in couples living in urban areas compared to those living in rural areas, but this difference was not statistically significant (p = 0.001). In a Polish multicentre study of 1517 individuals, 18.9% of couples were both contributory to poor reproductive potential. Idiopathic aetiologies were seen in 15.99% of cases [17]. Male factor infertility accounted for 55.73% of cases. A cross-sectional population survey on fertility status conducted by Datta et al. in Britain was applied to 8869 women and 6293 men aged 16–74 years [18]. Approximately 10% of men reported infertility in this group. The prevalence of infertility was noted to be higher among individuals who postponed their parenthood. In addition, 57.3% of the men seeking help for their infertility problems had better education and higher status in their occupations. In 2004, Bayasgalan et al. determined the clinical patterns and major causes of 430 infertile couples attending an infertility clinic in Mongolia [19]. Male factor infertility was present in 25.6% of cases. Obstructive azoospermia (8.4%) and acquired testicular damage (5.4%) showed higher prevalence compared to other causes. In India, an estimated 15–20 million couples suffered from infertility in 2009 [20].

More accurate infertility rates can be observed in developed countries, such as Australia, Europe and North America, compared to developing countries. These countries have organizations, such as the National Survey of Family Growth [21], Australian Institute for Health and Welfare [22] and European Association of Urology (EAU) [23], that provide the most detailed reporting of data available in infertility problems. Despite the figures, it is very difficult to determine an unbiased prevalence of male infertility within the global, regional or national populations due to the low methodological quality of evidence [24].

General Epidemiology of Genetic Infertility

Complex and multifactorial conditions can cause male infertility, and aetiologies of male infertility can be either acquired or congenital. Despite all exhaustive efforts to determine the exact nature of the causes of male infertility, many are still diagnosed with idiopathic male infertility. A number of these causes can be explained by genetic abnormalities [25]. The search for "hidden" genetic factors was widely inefficacious in detecting recurrent genetic factors with potential clinical applications [26].

Genetic anomalies, including numerical and structural chromosomal abnormalities, have been linked with unexplained oligozoospermia and azoospermia [27]. The prevalence of chromosomal abnormalities is indirectly proportional to sperm concentration [28]. These are seen less in men with normal sperm concentrations (<1%) compared to men with oligozoospermia (5%) or azoospermia (10–15%).

Azoospermia can be identified in 15% of men with poor reproductive potential [29]. This can be classified as obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). Forty percent of azoospermic men present as OA, while the remaining 60% are NOA, and the latter is frequently associated with testicular failure. Some of the causes of NOA remain unknown. This might be secondary to genetic abnormalities. Genetic anomalies causing azoospermia can be grouped into two large categories comprised of chromosomal and non-chromosomal [30]. Chromosomal anomalies can be further subdivided into structural abnormalities, including chromosomal inversions and translocations and Y chromosome microdeletions (YCMD), and numerical abnormalities (aneuploidy). Non-chromosomal anomalies include sperm mitochondrial genome defects and epigenetic alterations of the genome. A higher incidence of aneuploidy can be observed in men with NOA. In a chromosome analysis of azoospermic men undergoing ICSI, the overall aneuploidy rate of 11.4% ($p = 0.0001$) in men with NOA was significantly higher than the 1.8% rate demonstrated in epididymal sperm from men with OA and the 1.5% rate detected in ejaculated sperm [31]. Cytogenetic analysis is significant in male infertility, as suggested by the frequency of chromosomal abnormalities in these infertile men, especially if the couple will undergo ART [32].

Some epidemiological studies are available on the genetic basis leading to the poor reproductive potential of male infertility. In an earlier review of pooled data from 11 surveys of 9766 men with severe oligozoospermia and azoospermia, the incidence of chromosomal abnormalities was 5.8% [33]. Autosomal abnormalities were detected in 1.5% of this combined population of men. On the other hand, sex chromosome anomalies were higher in azoospermic and infertile men, with an incidence of 4.2%. In another series of studies of 94,465 newborn male infants, chromosomal abnormalities were detected in 0.38% ($n = 366$) compared to phenotypically normal newborns. Of those with anomalies, 0.25% ($n = 232$) were diagnosed with autosomal defects, while 0.14% had sex chromosomal abnormalities ($n = 131$). In another study, a cytogenetic investigation in France was performed in infertile men with low sperm concentration over a period of 25 years [34]. In total, 13,154 men

were clinically examined and biologically investigated to determine the genetic abnormalities of their poor reproductive outcomes. Somatic cytogenetic abnormalities were detected in 7.7% of these infertile men. The highest abnormalities were observed in men with NOA (16.7%, $n = 108$), followed by men with sperm counts $<5 \times 10^6$/ml (9.7%, $n = 63$), 5–10 $\times 10^6$/ml (4.3%, $n = 27$) and > 10–20 $\times 10^6$/ml (0.5%, $n = 3$). Sex chromosomal abnormalities were demonstrated more often in patients with NOA (77.1%, $p < 0.001$) compared to men with oligozoospermia and obstructive azoospermia. Nagvenkar et al. determined the chromosomal constitution of 88 infertile Indian men, comprising 42 azoospermic men and 46 severe oligozoospermic men undergoing ICSI [35]. Overall, 10.2% had chromosomal abnormalities; higher rates were observed in men with azoospermia (14.3%) compared to men with severe oligozoospermia (6.5%). Robertsonian translocation was detected in one patient. In a study by Samli et al., 819 men with azoospermia ($n = 383$) and oligozoospermia ($n = 436$) were evaluated for genetic factor as their cause of their infertility [28]. Chromosomal abnormalities were diagnosed in 12% ($n = 47$) of azoospermic men and 4% ($n = 20$) of oligozoospermic men. In the azoospermia group, 19% ($n = 9$) of the chromosomal anomalies were due to autosomal abnormalities, while 80% ($n = 38$) were secondary to gonosomal abnormalities. Among 2710 infertile couples who were candidates for ART in Italy, 74 aberrant karyotypes were found [36]. This corresponded to 1.5% ($n = 40$) in men. Most of the observed chromosomal abnormalities (2.2%) were from men whose partners underwent ICSI; this was higher compared to men whose partners underwent intrauterine insemination (IUI) (0.3%) and IVF (1.1%). In another study in Brazil, Mafra et al. made a retrospective genetic assessment of 143 infertile men composed of 100 men with severe oligozoospermia and 43 men with NOA [37]. Genetic abnormalities were detected in 18.8% of all infertile men. Nine men had chromosomal abnormalities, of which four were from the azoospermia group and five from the oligozoospermia group. YCMD was found in 4.2% of men, all of which were detected in the azoospermia and oligozoospermia groups. A study in the Middle East showed a 9.59% incidence of chromosomal abnormalities in 511 patients [38]. Nineteen (10.6%) of 179 Qatari men had chromosomal abnormalities, while 30 (9.04%) of 332 non-Qatari men were similarly diagnosed. Chromosomal abnormalities were diagnosed in 10.78% of azoospermic men, while they were detected in 7.5% of oligozoospermic men. Overall, the most common chromosomal abnormality was Klinefelter syndrome, which was present in 19 men. This was followed by 13 men with YCMD [azoospermia factor (AZF)a = 1; AZFb and c = 5; AZFc = 7]. Both Robertsonian translocation and reciprocal translocation were demonstrated in six men in each abnormality. Five men had other chromosomal aberrations. Punab et al. conducted a 9-year monocentre, prospective, clinical-epidemiological study of 8518 infertile men from Estonia with reduced total sperm count (<39 million per ejaculate) in at least 2 consecutive semen analyses [39]. Among these male partners of infertile couples, 20.4% ($n = 1737$) had severe male factor infertility. In 40 per cent ($n = 695$) of cases, the primary cause of infertility was determined, but 60% ($n = 1042$) remained idiopathic. In patients with known genetic aetiologies ($n = 135$), 87.4% had extreme infertility comprised of azoospermia, cryptozoospermia and

aspermia. The prevalence of congenital abnormalities was not clearly associated with the severity of deranged spermatogenesis. In Eastern China, Xie et al. reviewed the cytogenetic results from 912 men with NOA ($n = 534$) and severe oligozoospermia ($n = 378$), while 215 normozoospermic men served as the control group [40]. Genetic anomalies were detected in 22.6% ($n = 206$) of cases. Among these, 27.35% ($n = 146$) of azoospermic men and 15.9% ($n = 60$) of oligozoospermic men had genetic abnormalities. Four (1.9%) men from the control group and 138 (25.8%) men from the NOA group had chromosomal abnormalities. The NOA group had a higher frequency of the 47,XXY karyotype compared to the oligozoospermic group (8% vs 1.1%, respectively). Likewise, a higher incidence of YCMD was observed in the NOA group (17.8% vs 13.2%, respectively).

The main genetic variants leading to male infertility are chromosomal alterations, inversions, translocation, Y chromosome microdeletions and gene mutations [41].

Epidemiology of Specific Genetic Abnormalities

Klinefelter Syndrome

Klinefelter syndrome (KS) is considered the most common chromosomal aneuploidy in infertile men with testicular failure [42]. This is more frequently seen in men with azoospermia, with a prevalence of 10%, and severe oligozoospermia, with a prevalence of 0.7%. Only 12% of patients with KS are detected prenatally, while diagnosis during their childhood and adolescence is approximately 25%; more than half of them will be left undiagnosed during their lifetime [43]. The classic form 47,XXY constitutes 80–90% of all cases of KS [44]. Of the other variants of KS, 48,XXYY occurs more frequently in 1:18,000–1:40,000 male births [45] compared to other forms, such as 48,XXXY and 49,XXXXY [46], which appear in 1:50,000 and 1:85,000–1:100,000 male births, respectively.

In Denmark, all cytogenetic examinations have been registered in the central registry since 1961 [47]. Of 76,526 prenatal examinations, 163 foetuses were detected with the KS karyotype. This resulted in a prevalence of 213 per 100,000 males. Among 2,480,858 males born between 1931 and 2000, postnatal diagnosis of KS was described in 696 boys and men. Among boys aged 10–14 years, the prevalence of diagnosed KS was 14.2 per 100,000 males, while a prevalence of 35–40 per 100,000 was observed in males aged 25–54 years. In America, a newborn screening for methylated FMR1 DNA to test for KS was performed in 36,124 newborn males [48]. In total, 57 were diagnosed with KS, showing an incidence of 1 in 633 newborn males. Of 16,252 samples from white males, 27 had KS. Of 10,979 samples from African Americans, 20 had KS. Three newborn males out of 5396 samples from Hispanic males and 847 Asian males were diagnosed with KS. In the Middle East, KS was diagnosed in 19 of 511 men [38]. The frequency of these genetic abnormalities increases with the severity of the spermatogenic defect.

A meta-analysis on sperm recovery and ICSI outcomes on men with KS was performed by Corona et al. in 37 trials comprising 1248 patients. An overall 44% sperm retrieval rate was detected per TESE cycle [49]. Similar results were observed for the biochemical pregnancy rate (43%) and live birth rate (43%).

Y Chromosome Microdeletions

YCMD is the second most common genetic cause of male factor infertility after KS [50]. The Y chromosome contains different genes that are important for the development of testis and spermatogenesis in humans [51]. The long arm of the Y chromosome (Yq) is susceptible to intrachromosomal deletions, and AZF microdeletions occur in infertile men. YCMD can occur in distinct variations, including AZFa, AZFb and AZFc. In the general population, Yq microdeletions are estimated to occur in 1:4000 men. However, in infertile men, their frequency is 1:12. The worldwide prevalence of AZF microdeletions in infertile men is 7% based on an analysis of more than 30,000 chromosomes.

YCMD is usually seen in men with severe oligozoospermia and azoospermia. In a meta-analysis by Yousefi-Razin et al. among Iranian infertile men, the frequency of Yq microdeletions was 12.1%, particularly among those with severe oligozoospermia and azoospermia [52]. Johnson et al. determined the threshold sperm concentration for genetic analysis of YCMD in 1473 infertile men in a multi-ethnic urban population in the United Kingdom [53]. In this study, the prevalence of microdeletions was 4%. Among men with AZF microdeletions, none of them had a sperm concentration more than 0.5×10^6/ml. Using this lowered sperm concentration threshold, a high sensitivity of 100% and increased specificity of 31% were observed.

Various AZF loci microdeletions differ among various populations according to global estimates. Chellat et al. determined the frequency of YCMD among 80 Algerian infertile men with azoospermia ($n = 49$) and oligoasthenoteratozoospermia ($n = 31$) and compared them to 20 fertile control men [54]. Only one man from the azoospermia group had AZFc microdeletions, suggesting an overall AZF deletion rate of 1.3% among this group of men. Among 1306 infertile Korean men who underwent molecular screening for YCMD, microdeletions were detected in 7.7% ($n = 101$) of cases [55]. AZFc microdeletions (54.4%) were the most commonly detected deletions, followed by AZFb (7.9%) and AZFa (5.0%). Among men with AZFc microdeletions, 38.4% had azoospermia, and 96.4% had oligozoospermia ($<1 \times 10^6$ sperm/ml, 85.2%; $1–5 \times 10^6$ sperm/ml, 11.1%; $5–20 \times 10^6$ sperm/ml, 3.7%). Out of 146 Tunisian infertile men with sperm counts $<5 \times 10^6$/ml (azoospermic =76; oligospermic = 70), 6.85% ($n = 10$) had AZF deletions [56]. Among azoospermic men, 11.84% ($n = 9$) had microdeletions. AZFc microdeletions were detected in eight azoospermic men and one oligozoospermic man. Three azoospermic men demonstrated AZFa, AZFB and AZFc microdeletions. In a study by Sen et al. in Indian populations, 3.4% ($n = 56$) of 1636 infertile men had Yq microdele-

tions [57]. This increased to 5.8% (n = 215) when additional data from published studies in the Indian population were included, constituting a total of 3647 cases. AZFc microdeletions (46.6%) were the most commonly observed, particularly in azoospermic men. Compared to Western populations, the Indian population had a lower frequency of Yq microdeletions.

AZFc is the most commonly deleted locus in male infertility, accounting for 60–70% of cases. This is followed by microdeletions of AZFa (0.5–4%), AZFb (1–5%) and AZFb+c (1–3%) [58]. Bansal et al. studied the AZFc region of the Y chromosome for complete (b2/b4) and partial microdeletions (gr/gr, b1/b3, b2/b3) [59]. This involved 822 infertile men and 255 proven fertile men. Higher cases of partial AZFc microdeletions were observed compared to complete deletions (6.20% vs 0.97%). gr/gr (5.84%) was most commonly seen in partial deletions. Although no significant difference was observed, men with gr/gr deletions had lower sperm counts compared to those without the deletions (54.20 ± 57.45 million/ml vs 72.49 ± 60.06 million/ml, p = 0.071). In addition, men with gr/gr deletions were at significantly higher risk of poor reproductive potential (OR = 1.821, 95% CI = 1.39–2.37, p = 0.000).

In men with severe testicular histopathology, including hypospermatogenesis, maturation arrest and Sertoli cell-only syndrome, 22–55% may harbour these microdeletions [59].

Congenital Bilateral Absence of Vas Deferens

Cystic fibrosis (CF) is the most commonly occurring autosomal recessive disorder in 1:1600 individuals from Northern European descent/non-Hispanic white populations [60]. Anomalies in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which controls the elevation of sweat sodium chloride concentrations through the adenosine monophosphate (AMP) pathway and regulation of the exocrine epithelial cell tubal secretion consistency, can result in CF. The Cystic Fibrosis Mutation Database has identified more than 2000 CFTR mutations [61].

Congenital bilateral absence of the vas deferens (CBAVD) is strongly related to cystic fibrosis (CF) [62]. The CFTR gene, which is located on chromosome 7, is mutated in 60–90% of men with CBAVD [8, 63]. Men with this type of abnormality can either have two mild mutations in the CFTR gene or a combination of mild and severe mutations [8]. F50del is considered the most severe CFTR gene mutation, occurring in 60–70% of men with CBAVD [8]. Mutations of the CFTR gene may still be undetectable in 25% of men with CBAVD despite the completeness of CFTR gene screening. However, using comprehensive and rapid genotyping of mutations and haplotypes combined with searches for rare large realignments, 87.9% of CFTR defects can be detected in men with CBAVD [64]. CBAVD can be observed in 4–7% of azoospermic men and 25% of men with obstructive azoospermia [65].

Among the 27,177 cystic fibrosis chromosomal analyses from 29 European countries and 3 countries from North Africa, Estivill et al. studied the geographic

distribution of 272 cystic fibrosis mutations [66]. The most common severe mutation encountered was delta F508, comprising 66.8% of the cases. Individuals from Denmark had the highest frequency of delta F508 mutations, while individuals from Algeria had the lowest (26.3%). Overall, 217 mutations were uncommon, with less than 1% frequency, while 55 mutations were common in 1 or more areas of Europe. On the other hand, the 5 T variant in intron 8 is the most common mild mutation in CBAVD [67].

In earlier studies by Kuligowska et al., transrectal ultrasound was performed in 276 infertile men with low semen volume and azoospermia [68]. There was an absence of anatomic abnormalities in 25.4% ($n = 70$) of cases. CBAVD was detected in 34.1% ($n = 94$) of men, while 11.2% ($n = 31$) were diagnosed with unilateral absence of the vas deferens.

Congenital unilateral absence of the vas deferens (CUAVD) is a different disease entity compared to CBAVD that results from embryologic Wolffian duct aberrancy [69]. Renal agenesis is usually seen in men with CUAVD. There is a 20% rate of CUAVD among men with unilateral renal genesis. On the other hand, men with CUAVD show a 79% rate of unilateral renal agenesis [62, 70]. If unilateral renal agenesis appears in CBAVD, this might be secondary to abnormal development of the entire mesonephric duct at a very early stage in the development of the embryo rather than a CF mutation [71].

47,XYY

Another chromosomal aneuploidy is XYY, which occurs in approximately 1:1000 live male newborns [72]. This happens due to nondisjunction at meiosis II, producing an extra Y chromosome. Men with 47,XYY syndrome have a wide variety of clinical presentations. These men are noted to have decreased fertility potential. Kim et al. presented three men with 47,XYY syndrome with varying degrees of oligozoospermia [73]. Most of these men had a normal phenotype; however, they were at greater risk for behavioural difficulties, learning disability, delayed development in speech and language and tall physical makeup [74].

In the Danish Cytogenetic Central Registry, 208 men were identified with 47,XYY from 1968 to 2008 [75]. The average prevalence was 14.2 per 100,000, and their median age at the time of diagnosis was 17.1 years. Shorter lifespans were also observed compared to men with normal karyotypes. The median age of survival was statistically less by approximately 10.4 years compared to the control group (67.5 years vs 77.9 years, respectively, $p < 0.0001$). In Iran, 37 cases of infertile men with 47, XYY were identified in a retrospective study [76]. Mosaicism was observed in 13 men, while 24 men had non-mosaicism. Among men with non-mosaic patterns, 9 had azoospermia, and 15 had oligozoospermia. Secondary infertility was demonstrated in two of the non-mosaic and three of the mosaic men.

Since this can be missed easily due to its wide spectrum of clinical presentations, accurate detection of this constitutional karyotype will help clinicians to correctly manage these men who undergo fertility evaluations [77].

46, XX

46,XX is a sex development disorder affecting 1:20,000 male newborns [78]. These individuals are phenotypically male and also display psychosexual identification as male. Their gonads are of a testicular pattern without evidence of ovarian tissues either grossly or microscopically. In addition, they have no female genital organs. The sex-determining region Y (SRY) is translocated to either end of the X chromosome or to an autosome [79].

In a review of records of patients evaluated for male fertility at two different institutions, six were identified to have 46,XX karyotypes. The mean age at diagnosis was 34.3 ± 4.5 years, and primary infertility was observed in all men. Semen analysis showed normal-volume azoospermia. Hormonal profiles of these men were consistent with hypergonadotropic hypogonadism. A literature search was done by Majzoub et al. that included 29 papers comprising 49 men with 46,XX [80]. Men with this disorder presented with sexual dysfunction (21%), reduced hair distribution (26.6%) and gynaecomastia (40%). In 83.7% of patients, the SRY gene was detected. The majority of these were translocated to a sex chromosome (95%) rather than an autosome (5%). In Turkey, ten men with 46,XX were identified upon evaluation in an infertility clinic between 2004 and 2015 [81]. The majority of cases (n = 8) had deletions of AZFa, AZFb and AZFc regions. Since most of them presented with hypergonadotropic hypogonadism, a negative effect of replacement therapies should be carefully observed. Lashkari et al. reported the genetic components of 8144 azoospermic and severe oligozoospermic Iranian men [82]. Among these men, 57 men were identified with 46,XX male sex reversal syndrome. Sixteen men presented with testosterone deficiencies, while 15 men were SRY-positive. In a recent publication from China of 144 males with 46,XX, hypergonadotropic hypogonadism was the main presentation of these men regardless of the availability of SRY [83]. Treatment options offered were limited to ART using donor sperm.

Kallmann Syndrome

Kallmann syndrome is one of the most common causes of hypogonadotropic hypogonadism. Mutations of the KAL1 gene are responsible for 30–70% of Kallmann syndrome cases. More than 25–50 genes were reported to cause idiopathic hypogonadotropic hypogonadism. Fifty per cent of all hereditary cases were mutations from these genes [84–86]. Hallmarks of this genetic disorder are anosmia and hypo-

gonadotropic hypogonadism [87]. Fifty per cent of patients with this disorder result from incomplete embryonic migration of gonadotropin-releasing hormone (GnRH) synthesizing neutrons. Approximately 10–20% of men will demonstrate spontaneous reproductive function recovery despite lifelong treatment [86].

Over a period of 5 years, 32 individuals (male = 26; female = 6) from 12 Jordanian and Palestinian families were evaluated for Kallmann syndrome [88]. Nineteen patients had olfactory tract agenesis discovered on magnetic resonance imaging (MRI). Anosmia was found in 27 patients, while 5 patients were hyposmic. Among the men affected with Kallmann syndrome, 73% had cryptorchidism, while 65% had microphallus. All other male patients demonstrated delayed puberty, hypogonadism and infertility.

Even though the genetic understanding of this syndrome is mostly unknown, mutations were identified in 5–10% of men with this condition [89]. Mutations in the KAL gene and AHC gene may lead to X-linked recessive hypogonadotropic hypogonadism.

Translocations

In men with severe male factor infertility, chromosomal translocations are the most commonly observed autosomal structural aberration [39]. Reciprocal translocation and Robertsonian translocation comprise this type of chromosomal anomaly. The carriers of Robertsonian translocation have a fusion of the long arms of two acrocentric chromosomes [90]. This rearrangement occurs when there is fusion of the complete long arms of two homologous or non-homologous acrocentric chromosomes [13–15, 21, 22, and] and a loss of the short arms of the translocated chromosomes [91]. The most commonly observed aberrations are t(13q;14q) and t(14q;21q). In earlier studies on populations of infertile men, approximately 0.8% were noted to be carriers of Robertsonian translocations, which was nine times higher than the general population. This rearrangement is found in 1:1000 newborns [42, 92]. Reciprocal translocation is a mutual exchange of chromosomal materials and segments between non-homologous chromosomes. This occurs in 0.7% of severely oligozoospermic and azoospermic men [93, 94].

There are stronger alterations of semen quality in men with Robertsonian translocations compared to reciprocal translocations. Both reciprocal and Robertsonian translocations are associated with a high rate of sperm aneuploidy.

Mayeur et al. performed a retrospective observational study over a period of 10 years on the association of these chromosomal translocations with sperm defects [91]. One hundred and five fertile men were compared to 81 men carrying reciprocal translocations and 63 men carrying Robertsonian translocations. Men with Robertsonian translocations (14.3%) were less frequently observed with normozoospermia compared to men with reciprocal translocations (39.5%). Men with Robertsonian translocations ($10.8 \pm 14.0 \times 10^6$/ml; 14.6 ± 12.7) had statistically lower

sperm concentrations and motility compared to the fertile group ($90.8 \pm 58.7 \times 10^6$/ml; 33.2 ± 6.6%) and reciprocal translocations ($49.0 \pm 50.1 \times 10^6$/ml; 22.1 ± 12.5%). Kim et al. reported a cytogenetic analysis of Korean patients suspected for chromosomal anomalies [95]. Among 4117 cases, 17.5% ($n = 721$) had chromosomal abnormalities. Aberrations in the structural chromosome of autosomes were found in 73% ($n = 527$) of cases. Translocations (43.6%) were the most frequent among this set of aberrations.

Inversions

Chromosomal inversions occur following two breaks in a chromosome and a subsequent 180° rotation of the segment before reinsertion [96]. In the general population, approximately 1–3% of individuals can demonstrate inversion, although the exact number remains unknown [95, 97, 98]. Carriers of this abnormality are at risk of infertility due to abnormal gamete production if there are an odd number of crossovers between the normal homologous chromosome and the inverted segment [96]; this will result in duplication or deletion. Chromosomal inversion can lead to infertility due to spermatogenic disturbances. These arise by the loops or eccentric fragments formed during meiosis. Suboptimal semen parameters can be observed in men with inversion carriers due to disrupted meiotic behaviour [99]. The risk of having a live birth with an unbalanced chromosomal karyotype is 1–10% [100]. A high degree of structural variability has been observed in human chromosome 9 [101].

The most common type of inversion is the pericentric inversion of chromosome 9, with an overall incidence of 1.98%, which is more frequently observed among those of African-American descent [102]. Male factor infertility is observed in 12% of patients with pericentric inversion [103].

Dana et al. performed a cytogenetic investigation of 900 infertile couples from Romania, of which 430 men had azoospermia and 76 oligozoospermia [104]. Among the individuals studied, 24 men (2.73%) had an inversion of chromosome 9. Mozdarani et al. studied 600 Iranian couples attending an infertility clinic. Fourteen men (4.69%) carrying a chromosome 9 inversion were detected [105]. During a 10-year period, Ait-Allah et al. reviewed the results of 652 cytogenetic studies on mid-trimester amniocentesis [102]. Pericentric inversion of chromosome 9 was detected in 27 cases. In this study, the incidence of inversion was 4.1%. In a Syrian population, 1 patient had an inversion out of 162 infertile men who underwent cytogenetic testing [106]. Pericentric inversion can present a variety of abnormal sperm parameters. In a study by Sasagawa et al. in six infertile men with pericentric inversion of chromosome 9 [107], semen analysis showed normozoospermia ($n = 1$), asthenozoospermia ($n = 3$), oligozoospermia ($n = 1$) and azoospermia ($n = 1$).

More reports and studies of chromosomal inversions are needed to assess their frequency and outcomes [98].

Significance and Difficulties in Epidemiological Studies in Genetic Male Infertility

Male infertility is a complex disease with a fundamental genetic basis. Due to the advent of ART, research on genetic aetiologies of male infertility has promptly expanded [108]. Genetic testing is now offered to infertile men to achieve appropriate diagnosis and treatment, including prognostic assessment. The detection of genetic factors in infertile men has become good practice for the applicable management of infertile partners.

A comprehensive male evaluation for all partners of infertile couples should be performed in order to uncover possible significant and treatable medical conditions before pursuing therapies with ART [109]. The difference of outcome of children born by ART and natural conception may be hampered by the genetic risks of infertile couples. Compared to the general neonatal population, ICSI foetal genetic testing showed an increase in de novo sex chromosomal aneuploidy (from 0.2% to 0.6%) and structural autosomal abnormalities (from 0.07% to 0.4%) and an increase in the number of inherited structural anomalies, mostly from the infertile male partner [110].

With the advancement of molecular genetic techniques, improvements in the diagnosis and treatment of male infertility have been observed. Clinical studies, karyotype investigation and biomarker research will equip clinicians to better and more deeply understand the aetiologies of male infertility. In an earlier retrospective study in 2 university-based male infertility clinics, 13 (1.1%) of 1236 patients had significant pathology [111]. One patient presented with azoospermia and bilateral small testis. Karyotyping revealed evidence of KS. The authors recommended that a comprehensive evaluation of the male partner by a male infertility specialist be provided for all couples presenting with fertility problems, including appropriate laboratory testing, such as genetic testing. Significant medical conditions are not frequently discovered during routine fertility assessments of the male partner. Since semen analysis can be easily performed in any laboratory, and no effective treatment exists for male factor infertility, comprehensive male factor infertility evaluations are usually bypassed, and most couples proceed directly to ART. This practice results in significant delays and even non-diagnosis of serious underlying medical conditions of men labelled as “infertile” and their potential children. In another study, Kolettis et al. determined the incidence of significant medical pathologies determined during a male infertility evaluation of 536 patients at 2 academic infertility practices [112]. Six per cent ($n = 33$) of men were identified as having significant anomalies. Genetic abnormalities were detected in 27 patients. Of these, 24 men had CF mutations, while the remaining 3 men had karyotypic abnormalities. Other pathologies discovered were testicular cancer ($n = 1$), prostate cancer ($n = 10$), diabetes mellitus ($n = 3$) and hypothyroidism ($n = 1$). Some doubt the usefulness of male infertility evaluations because good reproductive outcomes can be achieved even without this evaluation and testing. This practice may lead to significant underlying medical problems or genetic abnormalities being overlooked. Unfortunately,

bypassing male factor infertility evaluations and going straight to ART will not provide the exact aetiology of infertility. As a result, appropriate management is not offered to these infertile men.

Genetic testing is fundamental for clinical decision-making in the treatment of infertile men. It can spare one from unnecessary medical or surgical treatment [61]. However, there is a lack of national and international rules for the genetic approach to evaluate infertile couples [36]. According to the available guidelines on male infertility, karyotyping and Y chromosome microdeletion are requested if there is azoospermia or severe oligozoospermia [sperm count <5 million/ ml [113] or <10 million/ml [23]]. Despite the availability and proper clinical use of the proven genetic assays for male factor infertility, these can diagnose only 20% of cases [114].

The widespread utilization of sperm chromosome aneuploidy testing has been impeded by the technical nature of the procedure and the cost associated with fluorescence in situ hybridization (FISH) probes [114]. In addition, these analysis techniques are available at a limited number of reference laboratories. Genetic testing remains an underutilized assay that might be beneficial for infertile men and their partners [115]. Because of the financial burden of genetic testing, Khurana et al. developed a nomogramme using sperm concentration and motility, serum testosterone level and testicular volume to predict the probability of advising genetic testing [116]. They also performed a cost analysis using this model. Utilization of this nomogramme missed 15.4% of genetic anomalies. Using the optimal cut-off value of 13.8%, a direct cost savings of 45% were observed.

In 2006, the Human Genetics section of World Health Organization (WHO) released the implications of genetic testing services in developing countries [117]. Some of the reasons they cited as preventing its full implementation and development were poverty, few trained health professionals, less priority from policy makers given to genetic services and cultural and religious factors. There is also a lack of epidemiological data from genetic diseases. Thong et al. reported on the challenges of medical genetics in developing low- and middle-income countries in the Asia Pacific regions [118]. Due to limited birth defects or rare disease registries in this region from a lack of accurate data on genetic conditions, the development of necessary genetic services is hampered [119].

Overall, the epidemiology of male infertility is a challenging study for well-described reasons. Winter et al. narrated the problems encountered in this type of study [120]. Male infertility is not a reportable disease, and the majority of individuals who undergo treatment are predominantly on an outpatient basis. Paying out of pocket and a lack of insurance coverage impede these infertile couples from seeking medical help for their problems. There is an underestimation of the outcomes based on the nature of men's fecundity. The true statistical numbers have been weakly estimated in the available heterogeneous studies. A wide variety of influencing factors are hindering these studies to better characterize the true nature of male infertility and its global and regional incidence. These include race, country, geography and unique at-risk groups that are needed to arrive at the true value for this epidemiologic research.

Many infertile men are not being evaluated for infertility. This reflects a potential implication to reproductive goals and men's health. Comprehensive male factor infertility evaluation is crucial because correct male evaluation and genetic testing can discover conditions that impose a threat to the health of these infertile men and their potential children.

Conclusion

The inability to father a child is a global health concern. Genetic disorders are more frequently seen in idiopathic infertile men with severe oligozoospermia and azoospermia. Careful screening of these patients and referrals to infertility specialists for long-term follow-up and surveillance are warranted. The percentage of each type of male infertility varies in different geographic populations and in different available bodies of literature. The prevalence and patterns of aetiologies of infertility are diverse in all societies worldwide. Genetic testing can identify specific genetic conditions that could be transferred to the offspring through ART and can impact the ability to acquire sperm by different sperm retrieval techniques. Further genetic studies will continue to advance our knowledge in the clinical and biological domains. However, some uncommon genetic causes have heterogeneous phenotypes, and a potential lack of symptoms specific for a particular disorder may hinder diagnosis and treatment. As we continue to discover more about the genetics of male infertility, we will arrive at a better evaluation and subsequent appropriate management of these men with poor reproductive potential. Large-scale, prospective, epidemiological studies may enhance our understanding of the genetic disorders of male infertility.

Review Criteria

An extensive search of studies examining the epidemiology of genetic disorders in male infertility was performed using search engines, such as ScienceDirect, OVID, Google Scholar, PubMed and MEDLINE. The overall strategy for study identification and data extraction was based on the following keywords: "epidemiology", "male infertility", "genetic abnormalities", "chromosomal abnormalities", "azoospermia", "severe oligozoospermia", "Klinefelter syndrome", "Y chromosome microdeletions", "47,XYY", "46,XX", "congenital bilateral absence of vas deferens", "Kallmann syndrome", "Robertsonian translocation", "reciprocal translocation" and "inversion". Articles published in languages other than English were not considered.

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Chapter 6
Genetic Evaluation of Male Infertility

Khalid A. Fakhro, Amal Robay, Juan L. Rodriguez-Flores, and Ronald G. Crystal

Key Points
- To review what is known about genetics of male infertility
- To review advances in genetic investigation allowing higher-resolution patient investigation
- To review advances in bioinformatics and next-generation sequencing allowing more rapid assessment of patient cohorts
- To review shared and different genetic architecture underlying various types of infertility
- To suggest study designs for future cohort-based analysis which may illuminate novel pathways for diagnosis and treatment

Introduction

Male infertility affects ~7% of men worldwide, with a wide range of severity and presentation in the affected population, from mild treatable difficulties to complete absence of sperm production [1]. Infertility is a complex condition attributed to lifestyle, environment, and genetics, the latter playing a role in all forms of infertility

K. A. Fakhro
Department of Genetic Medicine, Weill Cornell Medicine – Qatar, Doha, Qatar

Human Genetics Department, Sidra Medicine, Doha, Qatar

A. Robay
Department of Genetic Medicine, Weill Cornell Medicine – Qatar, Doha, Qatar

J. L. Rodriguez-Flores · R. G. Crystal (✉)
Department of Genetic Medicine, Weill Cornell Medical College, New York, NY, USA
e-mail: rgcryst@med.cornell.edu

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[2], including quantitative, morphological, movement, hormonal, and obstructive abnormalities. Depending on the clinical findings, genetic screening may be recommended based on established guidelines [3, 4], for example, screening for Y chromosome deletions in azoospermia or channelopathies in motility defects. Nevertheless, despite improvements in molecular understanding of infertility, and despite the identification of hundreds of genes related to the condition, a substantial proportion of infertile males remain idiopathic with no genetic etiology established. Emerging research demonstrates that a proportion of these patients may benefit from next-generation sequencing to search for causes beyond known genes.

We present a description of the recommended genetic testing for males with different types of infertility, followed by discussion of advances in genome-wide evaluation and their utility at finding new genes causing infertility. Altogether, genetic causes currently account for ~25% of idiopathic patients, leaving a significant gap in diagnosis to be closed in the future [2].

Genetic Evaluation of Male Infertility

Genetic evaluation is indicated for specific patients with male infertility primarily based on the type of abnormality found. The main types of infertility that undergo genetic evaluation include (1) obstructive defects such as congenital absence of the vas deferens; (2) quantitative abnormalities such as oligospermia or azoospermia; (3) morphological abnormalities such as globozoospermia; and (4) motility defects such as asthenospermia. Traditionally, genetic investigation of infertile males relied primarily on low-resolution methods that attempted to identify gross chromosomal abnormalities in these patients. These included karyotyping and FISH.

Karyotyping is a technique through which the number and appearance of all chromosomes in an individual are assessed to determine if any carry a specific defect [5]. A patient's karyotype can be viewed under a light microscope, revealing large-scale defects such as missing or duplicated chromosomal segments or chromosomal fusions or aneuploidies. Classic examples of defects causing infertility that can be detected by karyotyping include Klinefelter and Turner syndromes, as well as a wide range of Robertsonian translocations. These and other examples are discussed in more details below.

Fluorescence in situ hybridization (FISH) is a technique that is similar to karyotyping, but is effectively able to detect chromosomal abnormalities that occur at a size range that is small than a typical chromosomal band (i.e., would be missed by regular karyotyping). These can include translocations, inversions, fusions, or copy number gains or losses, usually affecting critical genes and causing disease. However, in contrast to regular karyotyping, FISH is considered a targeted method because it requires the use of a specific probe to bind target DNA sequences, whereas karyotyping, being probe-less, gives a general picture of gross chromosomal abnormalities. But one important advantage of FISH, in addition to detecting smaller abnormalities below the resolution range of karyotyping, is the availability of a

wide number of fluorescent labels, which makes FISH a highly accurate assessment method that can effectively be used to probe up to tens of loci all at once.

Although the testing for each of these categories is different, advances in next-generation sequencing, both in interpretation and cost, may transform clinical practice such that whole-genome sequencing is adopted as a single test for all infertile men at the point of care, improving diagnosis and treatment in the future.

Obstructive Conditions

In cases of azoospermia, it is important to establish if the absence of sperm is due to obstruction or spermatogenic impairment. Obstruction may be caused by external factors, or by inherent physiological factors, most prominently abnormalities of the epididymis or the seminal vesicle [6, 7]. In most cases, an ultrasound is suggested to rule out renal abnormalities, which may suggest different genetic causes related to mesonephric duct malformations during embryonic development [8]. In the absence of renal involvement, isolated congenital unilateral or bilateral absence of the vas deferens accounts for 25% of obstructive azoospermia patients [9]. Two genes are connected to this condition, most prominently the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) and the adhesion G-protein-coupled receptor G2 gene (*ADGRG2*).

Analysis of the *CFTR* gene is particularly challenging because the gene itself has more than 2100 known variants spread across 27 exons, and the frequency of these variants differs significantly based on ethnicity and geographical region (www.genet.sickkids.on.ca). Also, most patients with congenital bilateral absence of the vas deferens (CBAVD) do not have other classic cystic fibrosis symptoms, complicating interpretation of variant severity in the absence of adequately population-matched screened controls. Comprehensive genetic testing should therefore examine the whole gene (including possible splice variants) instead of targeting only the most prevalent exon(s)/variant(s) in a given geographical location [8, 10, 11].

The involvement of the X-linked *ADRG2* gene was only recently discovered by next-generation exome sequencing of patients with CBAVD who tested negative for CFTR variants [12]. Variants in this gene were found to account for up to 15% of CBAVD patients, and the physiological mechanism was demonstrated in mice, where *Adrg2* mutants had fluid accumulation in the testicular ducts that caused obstruction [13, 14].

Quantitative Conditions

Quantitative abnormalities account for the majority of infertile patients and range from mild reductions in count such as in oligospermia (<15 million/ml) to complete absence of spermatogenic production (non-obstructive azoospermia). Genetic

testing for quantitative abnormalities includes a wide range of options, reflecting improvements in genetic assessment of infertile males over time.

Y chromosome deletions

Deletions on the Y chromosome represent one of the most prevalent causes of quantitative spermatogenic impairment in men and are recommended in diagnostic settings for any males with quantitative abnormalities [15]. Along the Y chromosome, the most commonly deleted part is the AZF region (so named after their discovery of being deleted in azoospermic men [16]), which comprises four different sub-regions found deleted in patients: AZFa, AZFb, AZFb+c, and AZFc [15]. Together, these deletions appear in 5–10% of azoospermic men and in approximately 2% of oligospermic men, but only in 0.025% of the general population [15, 17]. In general, most pathogenic deletions remove more than one complete region, with each region in turn encompassing a number of candidate spermatogenesis genes that are completely lost due to the existence of only one copy of the Y chromosome [18, 19]. The exact role of deleted genes remains to be fully understood, for example, deletions in USP9Y in the AZFa region appear in both azoospermic and normospermic men [20, 21]. Similarly, deletions of the *gr/gr* region within AZFc region, which contains dosage-sensitive transcription units [22], can be pathogenic or not depending on the patient's ethnicity, e.g., in Caucasians, there is a fourfold increase risk of severe oligospermia in the presence of this deletion, whereas in Japanese and East Asian patients, the same deletion appears to be fixed in the population and not have an effect on spermatogenic quantity [18, 23].

In terms of clinical severity, deletions of the AZFa region lead to Sertoli cell-only syndrome, with complete absence of spermatogenesis and azoospermia. AZFb and AFZb+c total deletions present with Sertoli cell-only syndrome as well as spermatogenic arrest. However, isolated AZFc deletions as well as partial AZFa or AZFb deletions sometimes lead to residual sperm production, with phenotypes ranging from azoospermia to severe oligospermia [18]. Thus, from a diagnostic and prognostic standpoint, AZFc or partial deletion carriers may elect to undergo surgical sperm retrieval, whereas complete deletion patients are unlikely to ever retrieve sperm and would probably not benefit from surgical procedures. In all cases, it should be considered that even if a patient elects to undergo sperm retrieval, all male progeny would be obligate carriers of the same Y chromosome, unlike autosomal gene mutations or deletions which could be selected against while still electing to have male progeny by in vitro fertilization.

Numerical chromosomal abnormalities and translocations The most common chromosomal abnormalities related to quantitative abnormalities are those affecting the sex chromosomes and Robertsonian translocations [6, 24].

Sex chromosomes Klinefelter syndrome (47,XXY) is a condition resulting in non-obstructive azoospermia, usually accompanied by other abnormalities including hormonal dysregulation, metabolic and cognitive abnormalities, and sometimes autoimmune findings [25]. Genetically, Klinefelter syndrome is characterized by an extra X chromo-

some, and a concomitant increase in levels of the female hormones FSH and LH in plasma, which in turn cause hypogonadism and androgen deficiency. Kallmann syndrome affects 1 in 650 males [26] and accounts for up to 14% of non-obstructive azoospermia cases [6]. Other sex chromosomal abnormalities that are not as common as Kallmann syndrome include Jacob's syndrome (47,XYY), De la Chapelle syndrome (46,XX male), and mixed gonadal mosaicism syndromes (e.g., 45,X/46,XY) [1].

Non-sex chromosomes The majority of non-sex chromosome-affected infertile men suffer from Robertsonian translocations, the most common form of chromosomal rearrangement. In this syndrome, five acrocentric chromosomes (13, 14, 15, 21, and 22) break at their centrosome and end up with the long arms fused to each other [27]. Though Robertsonian translocations affect up to 1 in 1000 newborns, most are phenotypically normal due to availability of a second full copy of each of the fused chromosomes, conferring the full dosage of the two chromosomes during development, and thus no excess or haploinsufficiency of specific genes is experienced by the individual [28]. However, in patients with male infertility, Robertsonian translocations are observed mostly in conjunction with severe oligospermia, but also less frequently with azoospermia, and can vary by ethnicity [29, 30].

X chromosome deletions and duplications A number of studies have revealed a role for copy number variations (CNVs) on the X chromosome in patients with quantitative spermatogenic abnormalities. Due to their smaller sizes, these CNVs usually encompass a few genes only, making them candidates for controlling fertility in a dosage-dependent manner. There are currently four such genes that can be tested for deletions or duplications. First, *TEX11* has emerged as a strong candidate, being reported to harbor both deletions and protein-disrupting point mutations in human patients [31–33] and having been shown to cause meiotic arrest in mouse knockout models [34]. Second, the androgen receptor gene *AR* is another candidate with known point mutations causing spermatogenic failure due to hormonal dysregulation [35] and also with known copy number involvement; specifically, a CAG repeat expansion in exon 1 of the gene appears to be a prominent risk factor for cryptorchidism and infertility [36, 37]. Third, a recent multicenter study revealed recurrent deletions affecting the *MAGE9* gene appear to be present in up to 1% of patients with quantitative spermatogenic abnormalities [31, 38–41]. Those variants were independently validated in another patient cohort, suggesting it could also be worth screening in a diagnostic setting [42]. Finally, a recurrent duplication of the non-coding RNA *LINC00685* was shown to affect up to 1.5% of patients with quantitative abnormalities, causing both oligospermia and complete azoospermia [43]. While array-based screening for these genes could possibly reveal dosage-impacting alterations, some of the genes also have point mutations that have been identified in patients (discussed in more details in the single-gene section that follows), suggesting a more comprehensive diagnostic screening strategy such as next-generation sequencing that assesses both copy number and point mutations may be able to resolve these cases faster in the future.

Single-Gene Tests

While some initial successes were demonstrated through re-sequencing of candidate genes from mouse and other organisms, in human, single-gene testing has sometimes yielded contradictory results, which are altogether insufficient to lead to adoption in clinical testing. For example, many variants that were initially described may only confer risk, and only in a specific subpopulation. Further, these variants may simply be rare polymorphisms that were impossible to interpret further in the absence of additional functional testing or replication in additional patient cohorts [44, 45].

Nevertheless, the emergence of gene panels and exome sequencing has allowed the identification of a number of genes that can be adequately screened at present in diagnostic settings. These include *AR*, *CCDC155*, *DNAH6*, *MCM8*, *MEIOB*, *NANOS2*, *NPAS2*, *SPINK2*, *SPO11*, *SYCE1*, *TAF4B*, *TDRD9*, *TEX14*, *TEX15*, *WNK3*, and *ZMYND15*. These genes play important roles in meiosis, acrosome biogenesis, and DNA replication checkpoints and as cellular transcription factors. Consistent with a dose-dependent mechanism, homozygous mutations in these genes usually cause azoospermia, while heterozygous mutations appear to cause oligospermia [46–53]. However, the rarity of these mutations and the requirement for their homozygosity indicate they are unlikely to affect a large subset of sporadic patients, in particular those without a history of consanguinity, and thus testing for them may not yield clinical diagnostic value in such patient groups.

Spermatogenesis is a complex process involving coordinated interaction of genes in complex networks: in humans, over 1000 genes are testis-specific (GTEx; [54]), and from model organism studies, it is estimated that over 2000 genes are directly involved in spermatogenesis. Screening for each of these separately may have been unnecessarily laborious and outside the recommended guidelines of diagnostic testing; however, the emergence of next-generation technologies could transform the way that future testing for infertility is performed at the point of care.

Morphological Abnormalities

Genetic screening may be prudent for a range of subtypes of morphological spermatogenic abnormalities, including acephalic spermatozoa, globozoospermia, macrozoospermia, and multiple morphological abnormalities of the flagella (MMAF). Most patients with morphological abnormalities have recessive (biallelic) mutations in genes controlling sperm structure.

Three genes have been implicated in causing acephalic spermatozoa: *BRDT*, *SUN5*, and *TSGA10* [55–57]. *SUN5* has the strongest evidence, with patients carrying recessive mutations presenting with headless sperm in addition to sperm with abnormal head-to-tail connections [58, 59].

Patients with globozoospermia present with round-headed, acrosomeless spermatozoa. The lack of an acrosome results in fertilization that cannot occur naturally,

and even with intracytoplasmic sperm injection, artificial activation of oocytes would be necessary for fertilization to proceed. Globozoospermia is caused by mutations in the genes, *DPY19L2*, *PICK1*, *SPATA16*, and *ZPBP* [45], any of which could be tested diagnostically.

In contrast, macrozoospermia is almost exclusively (>80%) caused by mutations in the aurora kinase C (*AURKC)* gene, with the exact mutation differing by ethnicity [45, 60]. This gene is essential for cytokinesis, and therefore mutations in the gene cause large-headed, tetraploid spermatozoa with multiple flagella [61].

Finally, MMAF is a hybrid between morphological and motility abnormalities, with many sperm harboring structural defects to the flagellum that in turn impairs movement [62]. Approximately 30 to 40% of MMAF patients have a mutation in the *DNAH1* gene, encoding the axonemal inner dynein arm heavy chain, and end up presenting with immotile (9+0) flagella [63, 64]. In addition to the dynein gene, several other genes have been implicated in morphological sperm abnormalities in humans and may be screened for as well, including *AKAP3*, *AKAP4*, *CFAP43*, and *CFAP44* [65, 66].

Motility Abnormalities

In addition to impaired motility in the setting of morphological abnormalities, there are several isolated motility defects that affect fertility with a known genetic cause. The most common motility abnormality occurs in the setting of primary ciliary dyskinesia (PCD), where ultrastructural abnormalities in cilia result in spermatocytes that look grossly normal but are unable to move [67]. Over 30 genes are known to cause PCD in humans, with most patients having recessive mutations in these genes that are possible to identify by genetic testing [68, 69].

Another subset of motility defects results from mutations in the sperm cation channel CATSPER genes that disrupt the sperm's ability to undergo hyperactivated motility and successful fertilization. The genetic factor in these cases is recessive point mutations or deletions in the *CATSPER1* and *CATSPER2* genes. In some cases, men with CATSPER mutations have hearing loss [70–72].

Next-Generation Sequencing Technologies

Altogether, there are a wide range of genetic causes underlying different subtypes of infertility, yet despite the significant progress and options in diagnostic testing, a substantial proportion of infertile men remain idiopathic, lacking a molecular etiology to explain disease. The development of next-generation sequencing promises to rapidly change the landscape of these diseases and increase the number of genes which can be discovered if the use of this technology is effectively implemented in the clinic.

In contrast to the laborious process of single-gene mutation screening through exon-by-exon amplification and Sanger sequencing, next-generation sequencing enables the interrogation of large panels of genes in a single experiment and at a reasonable cost [73–75]. Next-generation sequencing can be broadly classified into two categories: targeted panels or whole genome. Targeted methods (aka "panel sequencing") include investigation of a group of genes usually selected on the basis of known disease associations or expanded to include genes within known disease pathways. In a sense, the most comprehensive panel approach is that of whole-exome sequencing, in which a "panel" containing all coding regions is captured and sequenced. Typical whole-exome sequencing panels also capture flanking regulatory regions, enabling assessment of mutations affecting conserved but non-coding *genic* elements, such as splice junctions and 3' and 5' UTR sequences [76].

Beyond whole-exome sequencing, whole-genome sequencing is used to discover additional variants in the entire human genome, including non-coding variation and copy number and unbalanced chromosomal variations [77, 78]. Given the number of human disorders related to structural variants, a single test that can assess both large and small genomic variations is sometimes preferable, and the cost of whole-genome sequencing is almost the same as the cost of running a microarray and whole-exome sequencing separately for the same individual.

One critical consideration of next-generation sequencing is that instruments generate massive amounts of data, requiring sophisticated computational infrastructure and tools (bioinformatics) to process and analyze the data. Regardless of the sequencing platform, generally bioinformatics pipelines share three common steps: read mapping, variant calling, and variant interpretation. Read mapping is the process by which the millions of short reads coming off the sequencers are mapped to a reference human genome by standard base alignment methods. After mapping, bases that differ from the reference are "called" as variants. Once variants are called, their putative effects can be determined based on the impact they appear to have on critical genomic segments, such as disrupting a conserved gene.

The most challenging aspect of next-generation sequencing bioinformatics is variant interpretation. It is at this step that the effects of each discovered variant are predicted, and thus their putative effect on disease is assigned. Variant interpretation not only depends on a priori genome annotation (e.g., the location of genes and amino acid are well-defined) but also on sequencing of large cohorts to be able to discriminate between candidate disease mutations and rare population-specific polymorphisms. As more populations get sequenced around the world, control databases will undoubtedly grow, improving variant interpretation in the future [79, 80].

Of key consideration in next-generation sequencing analysis is the large number of variant sites produced per individual (three to four million per person); while a small subset of these may affect genes with known function, the vast majority are variants of unknown significance whose interpretation and relevance to health and disease is entirely unclear [81]. Robust clinical implementation of next-generation sequencing should attempt to deal with such variants accordingly, bearing in mind that while many are uninterpretable today, they may turn up meaning in the future and may therefore be relevant to the subject's health and should not be discarded.

The fact that the field is constantly undergoing discovery, with >200 new genes and thousands of variants being linked to diseases each year in humans and many more in model organisms [62, 82], presents a critical challenge of keeping annotation databases up to date. Thus, the new mantra is to "sequence once, interrogate often" based on the premise that a patient's genome will not change over time and could be reassessed for causal variants periodically as annotations improve. This strategy would be useful in conditions such as male infertility, where the longevity of the data could allow re-interrogation of idiopathic cases as more genes become known in the literature. Such considerations need to be taken into account when designing clinical next-generation sequencing pipelines, to ensure that genetic testing of patients is comprehensive, accurate, and reproducible.

Successful Application of Next-Generation Sequencing to Male Infertility

As described earlier, there are many causes for male infertility, including genetic disorders (e.g., chromosomal anomalies or gene defects), hormonal causes, genital infection or trauma, varicocele, chemical or physical agents affecting spermatogenesis, and duct obstruction. Genetic anomalies have been reported in 2.2–10.8% of cases of male infertility and are higher in cases of severe quantitative infertility defects (azoospermia and severe oligozoospermia) [83]. However, in 30 to 40% of cases of male infertility, no cause can be identified by standard diagnostic criteria described earlier in this chapter, and these cases are labelled "idiopathic" [3]. In these cases, genetic abnormalities are still highly suspected though the genes in which they occur remain unknown.

The value of knowing the genetic mutation in these families is severalfold. First, it allows for additional screening in other males in the family to rule in/out infertility. Second, in the last few decades and with the advances in in vitro fertilization and introduction of intracytoplasmic sperm injection, severe male infertility cases with few sperm in semen or even cases of azoospermia with focal intra-testicular spermatogenesis could potentially father their own children. This highlighted the need for proper genetic diagnosis to avoid vertical transmission of genetic abnormalities or production of more unstable genetic defects in the newborn. Finally, it provides a window into the biology of spermatogenesis in humans, which could be eventually targetable in the long run by therapy or pharmacologic intervention. Thus, it is prudent for next-generation sequencing technology to enter the male urology clinic.

Suitability of Male infertility for Next-Generation Sequencing

Male infertility is a complex disorder where rare and common variants both play a role in disease. However, like other complex disorders, there will be Mendelian forms of infertility that could be quickly solved by next-generation sequencing.

Since infertility significantly affects reproductive fitness, it is suspected that causative variants will remain at low frequency in the population. However, one important difference between these variants and those that cause other rare, severe disorders is that these may be carried and passed down from females, and thus, their frequency may be higher than usually anticipated for rare diseases. Additionally, advances in in vitro fertilization may lead to successful transmission of disease-causing variants if they happen to be carried in the sperm used for fertilization.

One of the most significant challenges to studying the genomics of male infertility is the identification of suitable controls. Without detailed semen analysis, it is impossible to rule out subfertility, even in fertile men with a history of fathering at least one child, and thus their use as controls should be done with caution since one cannot know for sure that a proven father does not also carry gene mutations that lead to subtle defects in motility, sperm morphology, or reduced sperm count.

Advances in Male infertility due to Next-Generation Sequencing by Subtype

Quantitative Anomalies

The most studied of male infertility subtypes using next-generation sequencing are the quantitative abnormalities non-obstructive azoospermia and oligospermia. Altogether, 19 genes [29, 33] have been implicated in causing quantitative defects in spermatogenesis by next-generation sequencing technologies (Table 6.1). The initial study was in 2013, in which the investigators used next-generation sequencing to refine a GWAS signal they had previously identified [84]. In this study, five genes were interrogated around peak association signals on chromosomes 12 (*PEX10*, *PRMT6*, and *SOX5*) and 20 (*SIRPA* and *SIRPG*). Using custom capture followed by sequencing on Illumina's first-generation Solexa platform of 96 non-obstructive azoospermia subjects and 96 healthy controls, the authors identified 6 variants in 3 genes (*SIRPA*, *RISPG*, and *SOX5*) that appeared at different frequencies between cases in controls [84]. To verify which of these could be causal, the authors then screened these 6 SNPs in an additional 520 NOA subjects and 477 controls. This analysis replicated two SNPs, a protective variant in *SIRPA* (rs199733185) and a variant that increases risk for non-obstructive azoospermia in *SIRPG* (rs1048055) [84]. In a separate study, an association was also found between a SNP in *SIRPA* (rs3197744) by targeted panel sequencing of cases and controls, supporting the putative role of this gene in male infertility [85].

Subsequently, a number of studies have used next-generation sequencing to assess individuals with non-obstructive azoospermia. Ayhan et al. (2014) [46] investigated two unrelated consanguineous families with spermatogenic failure, the first with three azoospermic brothers and one oligospermic and the second with three azoospermic brothers. In this study, the investigators used a hybrid approach of

Table 6.1 Genetic variants discovered in infertile men by next-generation sequencing technologies

Infertility classification[a]	Gene identified	Reported alleles[b]	Study method[c]	Cohort[d]	Cohort size[e]	Number assessed[f]	Number with variant(s)[g]	**Ref**
Quantitative	ADGRG2	[c.A2968G (p.K990E)], [c.G1709A (p.C570Y)]	WES	Sporadic	18 cases	18	1	[12]
	CFTR	c.350G > A (p.Arg117His)	Panel	Sporadic	1112	1112	1	[87]
	DNAH6	c.C10413A (p.H3471Q)	WES	Familial	1 family	5	2	[52]
	DNMT3L	dup21q22.3, del21q22.3[h]	WGS	Sporadic	33 cases	33	2	[88]
	HLA-DQA1, HLA-DRB1	dup6p21.32[h]	WGS	Sporadic	33 cases	33	1	[88]
	MAGEB4	c.1041A > T (p.*347Cys-ext*24)	WES	Familial	1 family	3	2	[86]
	MEIOB	c.A191T (p.N64I)	WES	Familial	1 family	4	4	[52]
	NPAS2	chr2: 101592000 C > G (p.P455A)	Panel	Familial	2 families	6	3	[49]
	SIRPA	[c.*273G > T, c.697G > A (p.Val233Ile)]	Panel	Sporadic	1800	1376	29	[84, 85]
	SIRPG	c.*223T > G (3'UTR)	Panel	Sporadic	1184	1184	2	[84]
	SPINK2	c.56-3C > G (splice)	WES	Familial	1 family	2	2	[53]
	SYCE1	c.197-2 A > G (splice)	WES	Familial	1 family	6	2	[47]
	SYCP3	c.524_527del (p.Ile175Asnfs*8)	Panel	Sporadic	1112	1112	1	[87]
	TAF4B	c.1831C > T (p.R611X)	WES	Familial	2 families	2	1	[46]
	TDRD9	c.720_723 del TAGT (p.Ser241Profs*4)	Panel	Familial	2 families	17	5	[51]
	TEX14	c.2668-2678del (early stop codon)	WGS	Familial	1 family	2	2	[52]
	TEX15	c.2130T > G (p.Y710*)	WES	Familial	2 families	10	4	[48]

(continued)

Table 6.1 (continued)

Infertility classification[a]	Gene identified	Reported alleles[b]	Study method[c]	Cohort[d]	Cohort size[e]	Number assessed[f]	Number with variant(s)[g]	Ref
	ZMYND15	c.1520_1523delAACA (p.Lys507Serfs∗3)	WES	Familial	2 families	2	1	[46]
Morphological	BRDT	c.G2783A (p.G928D)	WES	Familial	1 family	1	1	[55]
	CEP135	c.A1364T (p.D455V)	WES	Familial	1 family	1	1	[91]
	DNAH1	[c.6253_6254del, c.11726_11727del (p.R2085fs, p.P3909fs)], [c.7377 + 1G > C ()], [c.A3836G, c.11726_11727del (p.K1279R, p.P3909fs)], [c.C12397T, c.11726_11727del (p.R4133C, p.P3909fs)], [c.5766-2A > G, c.G10630T (p.E3544X)], [c.C4115T,c.11726_11727del (p.T1372M, p.P3909fs)], [c.C6822G, c.G9850A (p.D2274E, p.E3284K)], [c.C7066T, c.11726_11727del (p.R2356W, p.P3909fs)], [c.C7066T, c.11726_11727del (p.R2356W, p.P3909fs)], [c.G2610A, c.G12287T (p.W870X, p.R4096L)], [c.G3108A, c.G5864A (p.W1036X, p.W1955X)], [c.T6212G, c.12200_12202del (p.L2071R, p.4067_4068del)]	WES	Sporadic	21 cases	21	12	[92]
	NPHP4	c.2044C > T (p.R682∗)	WES	Familial	1 family	2	2	[90]
	SUN5	[c.381delA (p.Val128Serfs∗7)], [c.824C > T (p.Thr275Met)], [c.381delA (p.Val128Serfs∗7)], [c.781G > A (p.Val261Met)], [c.216G > A (p.Trp72∗)], [c.1043A > T (p.Asn348Ile)], [c.425.1G > A/c.1043A > T (p.Asn348Ile)], [c.851C > G (p.Ser284∗)], [c.340G > A (p.Gly114Arg)], [c.824C > T (p.Thr275Met)], [c.1066C > T (p.Arg356Cys)], [c.485T > A (p.Met162Lys)]	Panel	Sporadic	15 cases	15	6	[58]

Motility	CFAP43	[c.2802T > A (p.Cys934∗)], [c.4132C > T (p.Arg1378∗)], [c.253C > T (p.Arg85Trp)], [c.3945_4431del (p.Ile1316Leufs∗10)], [c.386C > A (p.Ser129Tyr)]	WES	Sporadic	30 cases	30	3	[66]
	CFAP44	c.2005_2006delAT (p.Met669Valfs∗13)	WES	Sporadic	30 cases	30	1	[66]
	CFAP65	c.5341G > T (p.Glu1781∗)	WES	Sporadic	30 cases	30	1	[66]
	DNAH1	[c.8626-1G > A (splice)], [c.11726_11727delCT (p.Pro3909ArgfsTer33)], [c.8626-1G > A (splice)]	Panel	Sporadic	6 families and 38 cases	59	10	[63]
	SPAG17	c.G4343A (p.R1448Q)	Panel	Familial	2 families	7	2	[93]

[a]Quantitative anomalies include azoospermia and oligospermia; morphological anomalies include teratozoospermia, macrozoospermia, globozoospermia, and acephalic spermatozoa syndrome; motility anomalies include asthenospermia and flagellar abnormalities impairing movement

[b]For each gene, reported alleles are included in Human Genome Variation Society (HGVS) format [113] (for each allele, the putative effect on the cDNA and protein is included). Where more than one allele was observed, individual's alleles are grouped by square "[]" brackets

[c]For each gene and alleles, the method of variant discovery by next-generation sequencing, WGS whole-genome sequencing, WES whole-exome sequencing, panel panel-based sequencing of a pre-selected group of genes

[d]Cohort type studied, familial, family-based sequencing; sporadic, cases or case-control design

[e]Number of individuals recruited to the study

[f]Number of individuals assessed

[g]Number in whom the reported variant(s) was found

[h]Copy number variant allele discovered by next-generation sequencing

employing whole-exome sequencing after single nucleotide polymorphism genotyping, which allowed a selective focus on runs of homozygosity to identify the causative variant [46]. This search led to the identification of a different gene for each family, *TAF4B* and *ZMYND15*, both harboring recessive deleterious truncating mutations shared by all affected brothers within each family [46]. Notably, the same recessive variant was shared by the oligospermic brother, suggesting some variable penetrance and supporting the grouping of quantitative abnormalities in a single genetic category.

Moar-Sagie et al. [47] used whole-exome sequencing in a single patient with non-obstructive azoospermia to find a candidate homozygous splice site mutation in *SYCE1*, which was then discovered to segregate with the disease in the family, i.e., one affected brother shared the same homozygous mutation, but it was absent from the fertile siblings and in heterozygous state in carrier parents, who were consanguineous. Okutman et al. [48] discovered a recessive mutation in *TEX15* segregating with non-obstructive azoospermia in three affected siblings in a Turkish family, absent from the fertile brother and parents. Ramasamy et al. [49] discovered *NPAS2* mutations in three siblings with azoospermia in another consanguineous family from Turkey. Finally, Gershoni et al. [52] used a combination of whole-exome sequencing and whole-genome sequencing in different families to discover mutations in the genes *MEIOB*, *TEX14*, and *DNAH6* [52]. In all cases, the mutations segregated with the affected members within each family and were rare in control databases, making them prime candidates for causing infertility.

More recently, five studies published in 2017 used next-generation sequencing in non-obstructive azoospermia or oligospermia patients to uncover additional genes causative of quantitative sperm defects and male infertility. Four of these focused on multiplex consanguineous families, establishing segregation of recessive mutations in *SPINK2*, *MAGEB4*, *TDRD9*, and *ADGRG2* with non-obstructive azoospermia siblings but none in healthy males in the family [12, 51, 53, 86]. The fifth study devised a novel experimental approach to assess both single nucleotide variants and copy number changes in 107 genes associated with male infertility from the literature [87]. By using single molecular inversion probes targeting 4525 genomic regions on 21 chromosomes, the investigators were able to rapidly screen for mutations in these genes in 1138 azoospermic or oligospermic subjects [87]. While the authors found six infertile males with chromosomal anomalies and five with AZF region deletions, point mutations were only found in an additional six subjects, five with *CFTR* mutations and one with a mutation in *SYCP3*, further reinforcing the notion that male infertility is extremely genetically heterogeneous. Nevertheless, the investigators commented that the sensitivity of their assay (e.g., detecting chromosomal abnormalities in patients who had already been screened by microarrays) and the cost of running such a scalable platform make it ideal for introduction into clinical settings.

In an extension of next-generation sequencing utility to the detection of structural variation, a group of 33 patients with spermatogenic failure and unexplained azoospermia were assessed by whole-genome sequencing for copy number variants (CNVs) [88]. Twenty-seven patients had a total of 42 CNVs detected, ranging in

size from 40 kb to 2.38 Mb. While these CNVs were distributed across multiple chromosomes, and some overlapped known CNVs common in the database of genomic variants, there were three loci that were absent from the database of genomic variants and were shared by more than one azoospermic subject: 21q22.3, 6p21.32, and 13q11 each shared by two individuals. Only the first two of these were genic, affecting the *DNMT3L* gene and the *HLA-DRB1* and *HLA-DQA1* genes, respectively. While HLA class II genes have been generally implicated in infertility [89], these two genes had not. Evidence supporting *DNMT3L* gene involvement is stronger, and its role in spermatogenesis and spermatogenic impairment has been shown previously [11].

Morphological Anomalies

Morphological anomalies impairing fertility occur in different forms, affecting the head, the neck, and the tail of the sperm. The latter usually cause motility defects (detailed in the next section), whereas the former can be further subdivided into macrozoospermia, globozoospermia, acephalic spermatozoa syndrome, or dysplasia of the sperm fibrous sheath. In the era of next-generation sequencing, only five studies have been published to date in which such affected subjects were sequenced. In the first of these studies, Alazami and colleagues [90] used whole-exome sequencing in a family with asthenozoospermia, identifying a nonsense mutation in *NPHP4*. In another study, Sha et al. [91] sequenced a patient with flagellar abnormalities and discovered a recessive deleterious mutation in *CEP135*, a protein necessary for centriole biogenesis. The mutation caused infertility by forming protein aggregates in the centrosome and flagella. In a separate study, Li et al. [55] discovered a mutation in *BRDT* in a consanguineous patient with acephalic spermatozoa. The homozygous mutation, which alters a highly conserved residue in the *BRDT* protein, is rare in the sense that its functional study revealed it is a gain-of-function recessive mutation [55]. In this case, it is likely that the gain of function on a single allele, such as those carried by the fertile brother and father, was not sufficient to impair fertility. Moreover, in the largest study on acephalic spermatozoa syndrome, Zhu et al. [58] used whole-exome sequencing in two unrelated infertile men and uncovered protein-altering recessive mutations in SUN5, one individual with a homozygous variant and the other with compound heterozygous variants. This prompted Sanger sequencing of an additional 15 patients, of which 6 individuals had additional recessive mutations in this gene. Finally, in a study of 21 patients with dysplasia of the fibrous sheath, Sha et al. [92] identified 17 unique *DNAH1* mutations in 12 cases, including 1 homozygous and 16 compound heterozygous patients. These mutations segregated in the cases but not in unaffected family members, or a cohort of 50 ethnically matched fertile men. Using functional investigations in a subset of patients, the authors show that these subjects have diminished *DNAH1* levels and disorganized microtubule arrangements. Together, these studies demonstrate the power of next-generation sequencing in detecting causative variants in morphological sperm abnormalities.

Motility Anomalies

Investigation of motility anomalies using next-generation sequencing has identified five unique genes from four separate studies. Amiri-Yekta et al. [63] began by investigating ten subjects in six highly consanguineous families with flagellar abnormalities using whole-exome sequencing. Mutations in *DNAH1* were identified in two families and confirmed in one additional sibling from each affected family by Sanger sequencing [63]. Subsequently, the investigators screened an additional 38 subjects for the same founder mutation, identifying 1 more patient who shares this same mutation. Wang et al. [64] used whole-exome sequencing to identify an additional four consanguineous Chinese subjects with frameshift truncating mutations in *DNAH1*, further establishing the role of this gene in flagellar development and motility during spermatogenesis. Further, Xu et al. [93] identified homozygous mutations in two siblings of consanguineous parents with mutations affecting a highly conserved residue in *SPAG17* causing asthenospermia. Functional studies showed this mutation causes significantly decreased SPAG17 expression in the patients' spermatozoa, consistent with a functional role in motility.

Tang et al. [66] investigated 30 independent cases with motility defects due to flagellar abnormalities and identified additional recessive mutations (homozygous and compound heterozygous) in the 3 cilia- and flagella-associated protein (CFAP) genes, *CFAP43*, *CFAP44*, and *CFAP65* in 5 subjects. Subsequent engineering of knockout mice for two of these genes (in *Cfap43* and 44) using CRISPR resulted in motility and flagellar abnormalities similar to those observed in the human patients.

CBAVD and Y Chromosome Next-Generation Sequencing Studies

While congenital bilateral absence of the vas deferens (CBAVD) is usually caused by *CFTR* mutations, one recent study discovered mutations in the X-linked adhesion protein *ADGRG2* [14]. By sequencing the exomes of 12 *CFTR*-negative men, followed by re-sequencing the *ADGRG2* gene in 14 additional men with CBAVD, they discovered 4 hemizygous mutations all predicted to truncate ADGRG2. This is consistent with mouse studies in which male Adrg2 knockouts develop obstruction and therefore infertility. A study by Oud et al. [87] identified a patient with congenital unilateral absence of vas deferens (CUAVD) with a *CFTR* mutation, further expanding the phenotyping spectrum of cystic fibrosis transmembrane receptor-based obstructive infertility.

One of the major advantages of whole-genome sequencing is the ability to detect both small and large variants, including structural and copy number variants. Such approaches have been employed recently on the Y chromosome to achieve breakpoint resolution for CNVs [94, 95] although no new causative genes have been identified so far. The ultra-repetitive nature of the Y chromosome, which is rich in

repeated elements and segmental duplications [19], makes CNV detection challenging using whole-genome sequencing, in particular accurate mapping of short reads. This mapping uncertainty has the potential to create false calls along the Y chromosome, an issue that could be mitigated with long-read technologies; however, those technologies are currently expensive and therefore not suitable for routine implementation.

Thus, given the current challenges of CNV assignment, it is no surprise that most next-generation sequencing studies ignore the Y chromosome [96]. While recent efforts have begun to patch together Y chromosome structural rearrangements using next-generation sequencing, there have been no studies targeting infertile men to date. This represents an interesting opportunity for future investigation, further justifying the use of whole-genome sequencing for patient assessment instead of whole-exome sequencing or panel sequencing where possible.

Altogether, 23 studies have been published to date using next-generation sequencing to discover mutations in 28 genes causing a wide variety of male infertility. This number likely represents the proverbial tip of the iceberg, with >400 genes identified to cause spermatogenic impairment in mice [97–99] and up to 100 genes identified in humans in the pre-next-generation sequencing era (reviewed in [48, 100–102]). However, as the technology is adopted more readily in clinical and research centers, it has the potential to discover many more.

Conclusions

The development and deployment of next-generation sequencing technologies have the potential to transform clinical testing across a wide range of human conditions, and male infertility is no exception. The work done to date is a testament that while the investigation of infertility by modern sequencing technologies may have only recently started, it has many opportunities for future discovery. Incumbent upon the success of next-generation sequencing are improvements to bioinformatics algorithms and tools that help transform data into actionable knowledge. Current test offerings are advancing from small gene panels to complete genomes, and with these advances comes an increasing need for improved bioinformatics, including analytics, annotations, and robust workflows to deliver this information to the clinic.

The work we have reviewed has focused entirely on the use of next-generation sequencing to uncover genetic variants in male infertility. However, next-generation sequencing has now been adopted to uses outside of genomic investigations, including, for example, transcriptomics, epigenetics, and investigations of the microbiome [reviewed in [103–106]. While such efforts have already begun addressing problems pertinent to male infertility (e.g., sperm cell transcriptomics [107], single-sperm cell genotyping [108], spermatocyte methylation analysis [109], seminal microbiome profiling [110]), these efforts have not reached mainstream analysis of large cohorts of affected patients. In addition to next-generation sequencing-based approaches, work on spermatogenesis is flourishing with the use of metabolomics

and proteomics. Detection of protein modifications, including important histone modification such as phosphorylation, ubiquitination, sumoylation, or acetylation, can shed light on gene expression patterns with functional consequences on normal (and, by extension, abnormal) fertility. Similarly, studies investigating non-coding RNAs and microRNAs regulating spermatogenesis have been undergoing in males with or without infertility to discover biomarkers predictive of infertility [111, 112]. Thus, there is substantial room to harness next-generation sequencing technologies toward conceptual advances in this condition.

One of the major open questions is how can next-generation sequencing be beneficial to patients of infertility, especially considering the difficulty correcting germline mutations in already affected individuals. First, we believe that for many individuals, receiving a genetic diagnosis is far more meaningful than living with the idiopathic label. The former can lead to transforming the clinical discussion from focusing on what is wrong to where to go next, rather than living a stressful, drawn-out trial and error approach of implementing various remedies in the hope of conception. Second, the availability of a diagnostic mutation could illuminate a therapeutic pathway for partially restoring fertility. While the field is still in its early days with regard to genetic studies, the emerging picture of high levels of genetic heterogeneity make it well suited for stratification of patient populations into different potential therapy groups based on affected genes and pathways. Separately, studies of these pathways may shed light on novel intervention possibilities or opportunities to repurpose medications to improve fertility outcomes. At the very least, knowledge of the genetic mutation can be used during in vitro fertilization (IVF) and intracytoplasmic sperm injection to select sperm cells not carrying the same mutation for male progeny.

The next decade has the potential to be defining for male infertility in particular, and human diseases in general, with advances in next-generation sequencing promising to play a large part. For infertile patients, there will be a long road ahead from sample collection to deriving clinical utility; in many cases, due to the significant genetic heterogeneity, the utility from any given sample will not be evident until many years down the road, when other patients with insults in the same genetic pathways are discovered. Nevertheless, patient populations should be encouraged to participate in genetic research so that those goals may one day be achieved.

Review Criteria

A comprehensive search of PubMed was performed for the following terms:

- Male infertility, spermatogenesis, spermatogenic failure, azoospermia, oligospermia, asthenospermia, teratospermia, genetics of male infertility, next-generation sequencing male infertility, exome sequencing male infertility, and genome sequencing infertility.
- We restricted the search to papers published up to Dec 2018 and focusing on humans and removing duplicates from the different search terms.
- A total of 113 articles were finally included in this chapter.

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Part II
Impact of Genetics on the Sperm Cell

Chapter 7
Genetic Basis of Sperm Morphologic Defects: Head Defects and Body and Tail Defects

Vineet Malhotra

Key Points

- Association of sperm morphological abnormalities with male infertility has been established in past two decades; however, genetic basis of these morphologic defects is still evolving.
- Identification of genetic abnormalities responsible for sperm morphologic defects has therapeutic potential which will ultimately lead to better management of infertility problems in couples.
- Teratozoospermia, characterised by the presence of over 96% of spermatozoa with abnormal morphology, is subdivided into polymorphic and monomorphic teratozoospermia (macrozoospermia, globozoospermia and acephalic spermatozoa syndrome).
- Chromosomal abnormalities, Y chromosome microdeletions and monogenic disorders are the most known and frequent genetic influencers implicated in male infertility studies.
- A positive AURKC mutation diagnosis in macrozoospermic patients; genetic mutations in ZPBP, SPATA16 or DPY19L2 in patients with globozoospermia; mutations in AKAP3/ APKA4 and DNAH1 in patients with MMAF; and mutations in SUN5, SPATA6 and PMFBP1 in patients with acephalic spermatozoa syndrome were identified.

V. Malhotra (✉)
Department of Urology and Andrology, Diyos Hospital, New Delhi, Delhi, India
e-mail: vineet@diyoshospital.com

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Introduction

Worldwide, the childbearing capacity of couples is decreasing at an alarming rate with an increase in the number of infertile couples. Looking at an overgrowing population of India, everyone would think that childbearing for Indian couples should be an effortless task. However, this is not true. Data about prevalence of infertility has shown contrasting depiction against the national population figure. An estimated number of couples, who suffer from infertility every year, has reached 60–80 million cases worldwide. In India, it has reached 15–20 million, which accounts for approximately 25% of the global infertility problem [1].

Sperm morphology has been shown to have a critical role in the fertilisation process in mammals, especially humans. Hence, a defect in sperm morphology is considered as one of the most important causes of infertility in males [2–4]. Teratozoospermia is characterised by the presence of over 96% of spermatozoa with abnormal morphology [5]. To be considered morphologically normal, a spermatozoon should have a normal acrosome, an oval head between 5 and 6 μm long and 2.5 and 3.5 μm wide, a middle piece 4.0 to 5.0 μm long and a tail or flagellum about 50 μm long.

Teratozoospermia can be subdivided into two categories. An ejaculate presenting an excess of spermatozoa with more than one type of abnormality is considered polymorphic teratozoospermia. When all the spermatozoa display a unique abnormality, teratozoospermia is said to be monomorphic. Three forms of monomorphic teratozoospermia are recognised: macrozoospermia (also called macrocephalic sperm head syndrome), globozoospermia (also called round-headed sperm syndrome) and pinhead/acephalic spermatozoa syndrome.

Literature published over the past two decades suggests multifactorial aetiology of male infertility in humans. One of the pivotal factors among them is genetics, which is implicated in about 15–30% of male infertility causes [6]. Recognising and understanding how genetic abnormalities influence male infertility is the foremost agenda of most research groups worldwide. Chromosomal abnormalities, Y chromosome microdeletions and monogenic disorders are the most known and frequent genetic influencers implicated in male infertility studies [7].

In this chapter, we have tried to give a general overview of sperm structure and types of common sperm morphology defects, and the main focus of this chapter is to discuss the association of genetic abnormalities with abnormal sperm morphology that impacts male infertility.

A Look Into the Basics of Sperm Morphology

Structure and Function of Each Part of a Normal Sperm

A mature sperm (also called spermatozoa) consists of the following four parts (Fig. 7.1):

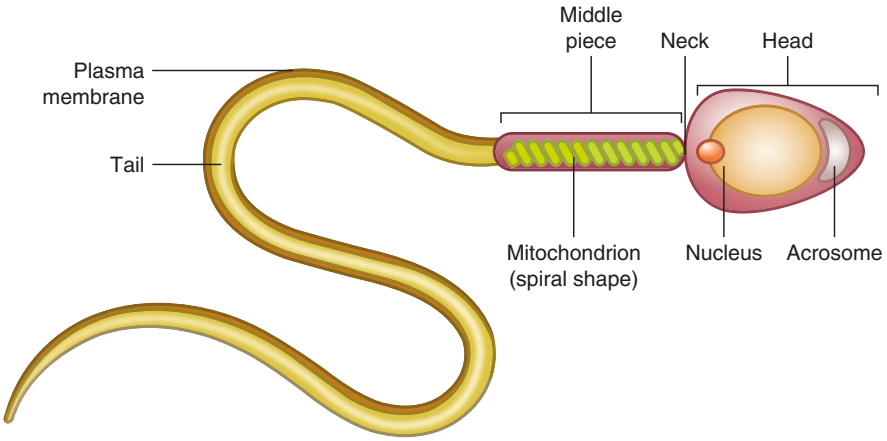

Fig. 7.1 Structure of sperm

I. *Head*

The head is oval in shape and is formed of two parts: (A) acrosome and (B) nucleus.

A. *Acrosome:* The acrosome is present at the anterior tip of the sperm and covers about 40–70% of the head area. It is formed from the Golgi complex during the process of spermatogenesis. It forms a cap-like structure known as the head cap. This occupies the space between the anterior half of the nucleus and the plasma membrane of the sperm tip. The acrosome is surrounded by a unit membrane and is composed of hydrolytic enzymes like acid phosphatase, hyaluronidase and others. These enzymes are also proteolytic in nature and help in dissolving the egg membrane through tissue damage and facilitating penetration of sperm into the egg membrane.

B. *Sperm nucleus:* The nucleus occupies most of the available space of the sperm head. The shape of the nucleus determines the shape of the sperm head. Structurally, it is enveloped by a nuclear membrane. The posterior part of the nuclear membrane (towards the body of the sperm) is somewhat depressed to accommodate the proximal centriole. The nucleus consists of DNA as well as basic proteins. There is no nucleolus or fluid content.

II. *Neck*

The neck forms the junction of posterior end of the head and anterior part of the middle piece and separates the middle piece of the sperm. The neck consists of two centrioles, viz. the proximal centriole and the distal centriole. Both these centrioles are situated very close and lie in the posterior depression of the sperm neck. The two centrioles enter the egg at the time of fertilisation along with the nucleus. These two centrioles are necessary to initiate division in the zygote. It is known that the centrioles help the zygotic division by forming the first mitotic spindle. The posterior or the distal centriole is responsible for the formation of the microtubules of the sperm tail.

III. *Middle Piece*

The middle piece of the sperm consists of the upper portion of the axial filament, and its structure has the same organisation as the axial filament of any flagellum. It has a pair of longitudinal fibres called beta fibres surrounded by a ring of nine pairs of longitudinal fibres called alpha fibres. In a human sperm, the alpha fibres of axial filament are accompanied on the outside by nine much thicker fibres called gamma fibres or coarse fibres. The alpha, beta and gamma fibres are the sites of various enzymes. These fibres are anchored to the distal centrioles and are surrounded by the mitochondria. Very often, the mitochondria are fused together and form a spiral sheet that surrounds the axonemal fibres. Around the periphery of the middle piece of the sperm, there is a thin sheet of cytoplasm mainly composed of microtubules. This layer is called manchette.

IV. *Tail*

The tail usually is the longest part of the sperm. In humans, it is about 55 μm long. It consists of two main parts: the principal piece and the end piece. The principal piece, which constitutes most of the length of tail, consists of the central core made up of axial filaments with a 9+2 arrangement (2 central, 9 peripheral). Surrounding this core is a fibrous tail sheath. In humans, two of the gamma fibres are fused with the surrounding ribs to form anterior and posterior columns extending throughout the length of the principal piece. This arrangement divides the principal piece into two functional compartments—one having three gamma fibres and the other having four. This symmetry is thought to help in a more powerful stroke of the tail in one direction. This is called the power stroke. The end piece is a small tapering portion of the tail containing only the axial filament surrounded by cytoplasm and plasma membrane [8].

Spermatogenesis and Spermiogenesis

Spermatogenesis and spermiogenesis are two stages that occur during the formation of sperms. Spermatogenesis is the complete process of the production of the sperm cells from the cells of the germinal epithelium of males. Spermiogenesis, on the other hand, is the final differentiation and maturation process of the spermatids into sperm cells [9].

Genetic Regulation of Spermatogenesis

Spermatogenesis is a complex process regulated by at least 2000 genes, and most of these are on autosomes with approximately 30 genes on the Y chromosome. While autosomal genes that regulate spermatogenesis are concerned with the regulation of metabolic process in both somatic and germ cells, Y chromosome genes regulate male reproductive processes [10]. Only a few human "spermatogenic genes" have been identified, though their precise function still remains unclear.

Genetic factors involved in male infertility are manifested as chromosomal disorders, monogenic disorders, multifactorial disorders and endocrine disorders of genetic origin [11, 12]. Chromosomal abnormalities are common in infertile men,

for example, in those who are 47XXY or have Klinefelter's syndrome [12]. Besides numerical abnormalities, which mainly involve sex chromosomes, structural abnormalities are usually autosomal [11].

Abnormal Sperm Morphology: Teratozoospermia

What Is Teratozoospermia?

Teratozoospermia is a semen alteration in which a large number of spermatozoa have abnormal morphology. It refers to semen with less than 4% morphologically normal spermatozoa. Optionally, the location of the defect can be specified as head defects, middle piece defects, sperm with cytoplasmic droplets and tail defects.

Defective spermatogenesis and some epididymal pathologies are commonly associated with an increased proportion of spermatozoa with abnormal shapes. The morphologic defects are usually mixed. Abnormal spermatozoa generally have a lower fertilising potential, depending on the types of anomalies, and may also have abnormal DNA. Morphologic defects have been associated with increased DNA fragmentation, an increased incidence of structural chromosomal aberrations, immature chromatin and aneuploidy [13].

Types of Sperm Morphology Defects (Head, Middle Piece and Tail Abnormalities)

According to the strict criteria defined by Kruger TF et al., a normal spermatozoon has specific dimensions with an oval head configuration featuring a smooth shape and no defects in middle piece and tail [14].

There is not enough information in literature about the types of defects in sperm morphology. Examples of commonly observed teratozoospermia include globozoospermia (round-headed sperm lacking an acrosome), macrocephaly (large-headed sperm), two-headed or two-tailed sperm, bent middle piece or the presence of a cytoplasmic droplet [15, 16]. Table 7.1 depicts a general comparison between normal and abnormal sperm morphology.

General Causes

The causes of teratozoospermia are still not entirely clear to the scientific community; however, some studies suggest the following factors may play a role in development of sperms with abnormal morphology:

Table 7.1 Comparison of normal and abnormal sperm morphology

Parts of sperm	Normal morphology	Abnormal morphology
Head	Regular oval shape, well-defined acrosome region without vacuoles and a volume of 40–70% of the head	Too big, too small, too thin and long, pear-shaped, round, amorphous, with acrosome vacuoles (>2 or more than 20%), post-acrosomal vacuoles, too small or too large acrosomes
Middle piece	Narrow, regular, about as long as the head. The main axis of the head and middle piece should be in line. Cytoplasmic droplets of the middle piece should be <30% of the head size	Asymmetric connection to the head, middle piece irregularly, too thick, bent or too thin. Cytoplasmic droplets >30%
Tail	The tail should be thinner than the middle piece, and the calibre should be uniform and the length about 10 times the length of the head length. The tail may be curved, but without abrupt kinks	Too short, multiple tails, kinks, irregular thickness, spiral-shaped

- Lifestyle.
- Semen infections.
- Pharmaceutical drugs that interact with testosterone, FSH and GnRH levels.
- Trauma or other testicular problems.
- Varicocele.
- Meningitis.
- Diabetes mellitus.
- High fever.
- Alcohol and drug abuse.

Diagnosis Criteria

The World Health Organization (WHO) has so far published several manuals in order to standardise semen analysis procedures, and WHO criteria have become widely accepted in sperm morphology examination at andrology laboratories all over the world. Furthermore, in 1990, Menkveld et al. showed that the assessment of sperm morphology by more stringent criteria, the so-called Tygerberg or strict criteria, enhances objectivity and decreases intra-laboratory variability [17].

Genetics of Male Infertility: Overview

About one-third of the infertility cases are unexplained (idiopathic male infertility), mostly due to our poor understanding of basic molecular mechanisms underlying male fertility [18]. It is estimated that genetic factors play a role in the pathogenesis

of 50% of men with idiopathic infertility [19]. Genetic factors implicated in male infertility are at every level of genetic information, from chromosomes to genes to nucleotides [6]. Chromosomal abnormalities, Y chromosome microdeletions, monogenic disorders [7] and genetic variability in several genes have been associated with male infertility [20–22].

Molecular and Genetic Basis of Teratozoospermia

Other phenotypes can be considered as pure teratozoospermia with 100% abnormal sperm and with a constant uniform pattern of anomalies, such as globozoospermia, large-headed multiflagellar spermatozoa or acephalic spermatozoa. To date, several genes have been shown to be involved in most of these specific teratozoospermia in humans. The identification and study of these genes shed a much-needed light on the physiopathology of teratozoospermia, as a prerequisite to improve patient management, to provide a basis for the development of therapeutic solutions tailored to the gene defect and to provide the patients with adequate genetic counselling and expected treatment outcome.

Several studies have identified genetic causes of various morphological abnormalities of spermatozoa, such as globozoospermia and macrocephalic sperm, when these abnormalities concern the vast majority of the sperm cells present [23]; association between genetic, lifestyle or environmental factors and abnormal sperm morphology have also been reported [24, 25].

Macrozoospermia

Patients with large-headed multiflagellar spermatozoa, macrozoospermia or macrocephalic sperm head syndrome present with a primary infertility characterised by the presence in the ejaculate of 100% abnormal spermatozoa with an oversized irregular head, abnormal middle piece and acrosome and multiple flagella. Ultrastructural study of such spermatozoa revealed a 3-fold increase in nuclear volume and on an average 3.6 flagella for each sperm head. This teratozoospermia is also generally associated with severe oligoasthenozoospermia [26–28].

Aneuploidy

Several studies using Feulgen-stained preparations [29], spermatocyte C-banding [30] and mainly fluorescence in situ hybridisation (FISH) analysis showed a high rate of polyploidy and aneuploidy in spermatozoa from men with macrozoospermia. Taken together, these observations provide evidence indicating that chromosome nondisjunction and/or cytokinesis defects occurring during the first, the second or both meiotic divisions are consistently associated with large-headed spermatozoa [27, 28, 31, 32].

A description of familial cases with consanguineous parents was suggestive of a genetic cause with an autosomal recessive inheritance. In 2007, a genome-wide low-density microsatellite analysis led to the identification of a common region of homozygosity in seven out of ten North African macrozoospermic patients, located in the terminal region of the long of chromosome 19. The AURKC gene, localised in the centre of this region, appeared as the ideal candidate because it was described as being expressed preferentially in male germ cells and to be involved in chromosomal segregation and cytokinesis, two functions that could explain the abnormal sperm morphology and cytogenetic content of large-headed spermatozoa (Fig. 7.2) [33]. Sequencing of the AURKC coding sequence allowed the identification of the same homozygous deletion (c.144delC) in all the 14 patients included in the study. This mutation introduces a frameshift resulting in a premature termination of translation and yielding a truncated protein lacking its conserved kinase domain [33]. It was later demonstrated that the mutated transcript is in fact degraded by the mechanism of nonsense-mediated mRNA decay, thus indicating that these patients do not even produce the truncated protein [34]. In another study, using flow cytometry in AURKC-mutated patients, Dieterich K et al. demonstrated that all spermatozoa had

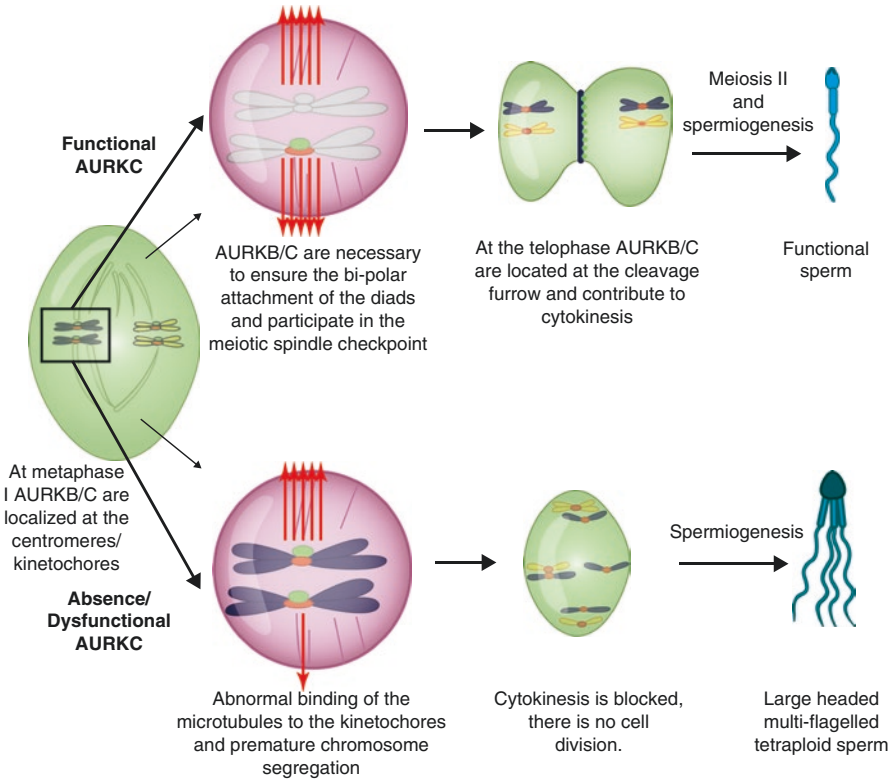

Fig. 7.2 AURKC controls meiosis and spermatocyte cytokinesis

a homogenous tetraploid DNA content indicating that the patients' germ cells undergo DNA synthesis but remain blocked without completing either of the two meiotic divisions [35].

AURKC belongs to the Aurora kinase family, which are highly evolutionarily conserved serine/threonine kinases playing a key role in the control of mitosis and meiosis. AURKC is expressed predominantly in testis, particularly in dividing spermatocytes, and in oocytes.

AURKC Mutations: The c.144delC Reigns Supreme

The c.144delC deletion accounts for 85% of the mutated alleles [36]. Other mutations that have been identified are p.C229Y, a novel missense mutation in exon 6 and c.144delC [35]; p.Y248∗, a new recurrent nonsense mutation that was found in ten unrelated individuals of European and North African origin [36]; and c436-2A.G, which leads to a shortened transcript devoid of exon 5 [34]. Overall, and excluding the study of Eloualid based on an unselected population of infertile men, a positive AURKC mutation diagnosis is found in between 50.8% and 100% of analysed macrozoospermic patients [37].

Globozoospermia

Globozoospermia is a rare (incidence 0.1%) and severe form of teratozoospermia characterised by the presence in the ejaculate of a large majority of round spermatozoa lacking the acrosome [15, 38, 39]. Globozoospermic sperms are unable to adhere and penetrate the zona pellucida, causing primary infertility [15]. The initial phenotype was divided into two subtypes: the globozoospermia type I, characterised by the complete lack of acrosome and acrosomal enzymes, and the globozoospermia type II, characterised by a round-headed phenotype due to a residual cytoplasmic droplet surrounding the sperm head and acrosome [40, 41]. However, this nomenclature is confusing and was subsequently often misemployed in the literature referring to patients with a homogeneous phenotype with 100% round-headed sperms (type I) or patients with a mosaic of normal and round-headed sperms (type II). The terms "total" or "partial" globozoospermia have been proposed and should be preferred to report the homogeneity of the "original" type I phenotype [42, 43], while the rarer, type II phenotype should be referred to as pseudo-globozoospermia.

Several familial cases of globozoospermia suggested a genetic contribution to this disorder [44–48]. Zona pellucida-binding protein (Zpbp or Zpbp1) and sperm acrosome-associated 1 (Spaca1) have an expression restricted to the testis. Zpbp and Spaca1 are integral acrosomal proteins but display different functions [49, 50]. ZPBD is localised in the acrosomal matrix and is involved in the binding and penetration of the sperm into the zona pellucida [51]. SPACA1 is a transmembrane protein located in the inner acrosomal membrane of spermatids and mature spermatozoa and plays an unidentified role in acrosomal morphogenesis and in sperm-egg

binding and fusion [50, 52]. Heterozygous missense and splicing mutations in ZPBP were described in patients presenting with abnormal sperm head morphology, but their involvement in the disease has not been clearly demonstrated [51]. Similarly, a homozygous missense mutation (G198A) in exon 13 of the PICK1 gene was identified in a Chinese family [53].

Homozygosity mapping using genome-wide scan analysis of a consanguineous Ashkenazi Jewish family with three globozoospermic brothers identified a homozygous mutation (c.848G.A) in SPATA16 (spermatogenesis-associated protein 16, previously named NYD-SP12) [54]. The SPATA16 protein localises to the Golgi apparatus and to the pro-acrosomal vesicles that fuse to form the acrosome during spermiogenesis [54, 55]. SPATA16 is highly expressed in the human testis and contains a conserved tetratricopeptide repeat (TPR) domain [56].

DPY19L2 belongs to a new family of transmembrane proteins of the nuclear envelope including, in mammals, four homologous proteins DPY19L1 to DPY19L4. These proteins are necessary to anchor the acrosome to the nucleus. In the absence of DPY19L2, the forming acrosome slowly separates from the nucleus before being removed from the sperm with the cytoplasm [57].

Few literature studies conclude that DPY19L2 deletion is the main cause of globozoospermia. Subsequently, three large studies confirmed the high prevalence of DPY19L2 gene alterations, ranging from 60% to 83.3% of the analysed patients in cohorts of globozoospermic patients from different geographic regions and with different ethnic backgrounds [58–60]. Two other publications strengthen this conclusion, reporting the presence of homozygous DPY19L2 deletion in patients from Macedonia and Algeria [61, 62]. Homozygous deletions represent 26.7–73.3% of the reported DPY19L2 mutations in the three largest studies [58–60].

DPY19L2 point mutations can be either missense mutations localised mainly in the central part of the DPY19L2 protein or nonsense/frameshift/splice site mutations resulting in truncated proteins [58–60]. Small deletions were also reported indicating that exon deletions are part of the mutational spectrum of the DPY19L2 gene [60]. A recurrent missense mutation in exon 8, p.Arg290His, was identified in several unrelated patients. This mutation changes a highly conserved arginine into a histidine and is predicted to be deleterious by multiple prediction tools [58–60].

Multiple Morphological Abnormalities of the Flagella (MMAF)

Multiple morphological abnormalities of the flagella (MMAF) come under abnormalities of the sperm tail. Morphological abnormalities of the sperm flagella leading to asthenozoospermia have been reported regularly since 1984 [63]. Chemes and colleagues carried out much of the early work on this phenotype and extensively studied the ultrastructure of the sperm flagella of affected men [64]. They

observed recurrent abnormalities of the fibrous sheath (FS) that defines the principal piece surrounding the axoneme and the outer dense fibres and consists of two longitudinal columns connected by circumferential ribs [65].

A genetic origin of MMAF was strongly suspected based on a family clustering reported in at least 20% of patients [66]. In 2005, BacettiB et al. first reported a partial deletion in the A kinase (PRKA) anchor protein 3 (AKAP3) and A kinase (PRKA) anchor protein 4 (AKAP4) genes in a patient presenting with short-tail spermatozoa [67]. Ultrastructural sperm evaluation showed MMAF and an altered axonemal structure lacking dynein arms and microtubular doublets including the central pair. A kinase (PRKA) anchor protein 3 (AKAP3) and A kinase (PRKA) anchor protein 4(AKAP4) encode two A kinase-anchoring proteins interacting with the regulatory subunits of cAMP-dependent protein kinase A. AKAP3 and AKAP4 are the most abundant structural proteins of the fibrous sheath. AKAP3 is involved in organising the basic structure of the fibrous sheath, while AKAP4 has a major role in completing fibrous sheath assembly [68]. Mouse models present strong evidence that Akap3 and Akap4 are involved in MMAF phenotype. Evidence of their implication in the human phenotype is weaker. A deletion of AKAP3 and AKAP4 was described in an MMAF patient, but it was only detected using conventional PCR, and the genomic breakpoints were not identified [67].

More recently, homozygous mutations in the DNAH1 gene were identified in several patients with MMAF [69]. DNAH1 encodes an axonemal inner arm dynein HC and is expressed in various tissues including the testis [70].

Acephalic Spermatozoa

Patients with acephalic spermatozoa syndrome are characterised by presence of headless spermatozoa (flagella) and a small proportion of sperm heads without flagella in their semen. It is a severe teratozoospermia that results in male infertility. Previous studies, including humans as well as animals, indicated that defects in formation of the connecting piece of spermatozoa during late spermiogenesis lead to acephalic spermatozoa [71]. Biallelic mutations in the SUN5 gene have been identified in these patients. Ten point mutations of SUN5 have been reported, including three nonsense mutations, six missense mutations and one intron mutation that may lead to splicing alterations [72]. SUN5 gene is located to the nuclear membrane at the tail/head junction in elongating spermatids and spermatozoa [73]. Similar to SUN5, SPATA6 now appears as a strong candidate for acephalic spermatozoa phenotype. Another study recently a homozygous nonsense mutation in the testis-specific gene PMFBP1. The disruption of Pmfbp1 in male mice led to infertility due to the production of acephalic spermatozoa and the disruption of PMFBP1's cooperation with SUN5 and SPATA6, which plays a role in connecting sperm head to the tail [74].

Conclusion

Previously published literature and ongoing research depict the need of better understanding of the genetic basis of infertility by clinicians as well as the public. Chromosomal abnormalities, Y chromosome microdeletions and monogenic disorders are the most known and frequent genetic influencers implicated in male infertility studies. This chapter emphasises on the genetic basis of sperm morphology defects and its association with male infertility. Most specific forms of sperm morphology abnormalities studied till date include macrozoospermia, globozoospermia and MMAF. Various studies suggest association between several genes and these abnormalities, which in turn impacts male infertility.

- Macrozoospermia: A high rate of polyploidy and aneuploidy in spermatozoa from men with macrozoospermia have been observed in several past studies. Taken together, these observations provide evidence indicating that chromosome nondisjunction and/or cytokinesis defects occurring during the first, the second or both meiotic divisions are consistently associated with large-headed spermatozoa. Furthermore, the AURKC gene appeared as the ideal candidate gene because of its expression in male germ cells and involvement in chromosomal segregation and cytokinesis. This could explain its role in the abnormal sperm morphology and cytogenetic content of large-headed spermatozoa.
- Globozoospermia: Several familial cases indicate a genetic contribution to this disorder. Heterozygous missense and splicing mutations in ZPBP were seen in patients presenting with abnormal sperm head morphology. Another study identified a homozygous mutation (c.848G.A) in SPATA16 in patients of globozoospermia. Further, few studies conclude that DPY19L2 deletion is the main cause of globozoospermia and observed a high prevalence of DPY19L2 gene alterations, ranging from 60% to 83.3% of analysed patients in cohorts of globozoospermic patients from different geographic regions and with different ethnic backgrounds.
- MMAF: A genetic origin of MMAF was strongly suspected based on few published studies. A partial deletion in the A kinase (PRKA) anchor protein 3 (AKAP3) and A kinase (PRKA) anchor protein 4 (AKAP4) genes in a patient were observed with short-tail spermatozoa. More recently, homozygous mutations in the DNAH1 gene, which encodes an axonemal inner arm dynein HC, were identified in several patients with MMAF.

Therefore, in conclusion, several genes were identified in most of these specific teratozoospermia in humans. The identification and study of these genes shed a much-needed light on the physiopathology of teratozoospermia, as a prerequisite to improve patient management, to provide a basis for the development of therapeutic solutions tailored to the gene defect and to provide the patients with adequate genetic counselling and expected treatment outcome.

Review Criteria
Literature search was performed using a combination of the following keywords: "sperm morphology defects", "male infertility", "genetic basis", "teratozoospermia", "globozoospermia", "spermatogenesis", "spermiogenesis", "sperm head abnormalities", "MMAF", "acephalic spermatozoa", and "molecular mechanism".

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Chapter 8
Mitochondrial Function and Male Infertility

David Fisher and Ralf Henkel

Key Points
- Optimal mitochondrial function is both central and crucial to male fertility, and therefore, understanding and monitoring of its function are expedient in treating male infertility.
- Mitochondrial membrane potential is a key variable in clinically understanding compromised sperm motility.
- Standard interpretation of the redox state of sperm may predict fertility.
- DNA analysis (nuclear and mitochondrial) may provide clarity as to the biological rationale for poor sperm motility.

Introduction

According to the endosymbiotic theory, mitochondria either developed from a symbiosis between aerobe bacteria or the incorporation of facultative anaerobe bacteria (symbiont) by methanogen archaea (host). In eukaryote cells, mitochondria are providing a major portion of the energy used by cells; these organelles are also called the powerhouse of the cell and are therefore critical hubs of a cell's metabolism. They are constructed with an outer and an inner membrane, which is highly compartmentalized into so-called cristae (Fig. 8.1). Between the outer and inner membrane is an intermembrane space, and the space within the inner membrane is called the matrix.

D. Fisher · R. Henkel (✉)
Department of Medical Bioscience, University of the Western Cape, Bellville, South Africa
e-mail: rhenkel@uwc.ac.za

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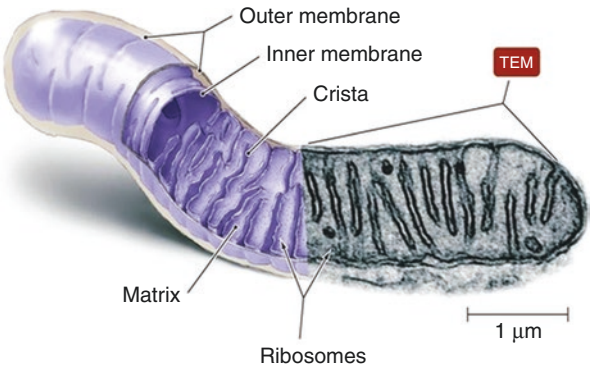


Fig. 8.1 The understanding of the structure of the mitochondria is essential to understand its function. The above diagram combines a schematic drawing and an electron transmission microscopy (TEM) rendition of the mitochondria

Male germ cells only have a very limited number of mitochondria, located in the midpiece of the sperm cell. Despite the fact that theories on sperm energy production are inconsistent (glycolysis or oxidative phosphorylation in the mitochondria), recent studies revealed the importance of the mitochondria in sperm function and successful fertilization. During evolution, the endosymbionts transferred more than 90% of their genome to the nucleus of the host cell. Yet, mitochondria still possess their own mitochondrial DNA (mt-DNA), which regulates and is regulated by nuclear gene activity. In addition to ATP synthase, mt-DNA encodes approximately 13 protein subunits of the mitochondrial electron transport chain as well as rRNA and tRNA units for the mitochondrial translation system [1]. In contrast to nuclear DNA (nDNA), mt-DNA is circular and not protected by the nuclear proteins histones and protamines. mt-DNA also replicates much faster than nDNA without proofreading and only very basic repair mechanisms available [2]. Mt-DNA is therefore prone to mutations and mitochondrial diseases including male infertility [3]. Therefore, this chapter tries to highlight the importance of mitochondria and their DNA for sperm function and the fertilization process.

Overview on Mitochondrial Function

Oxidative Phosphorylation

Oxidative phosphorylation (OxPhos) is essentially a metabolic process whereby the specialized arrangement of proteins bound to the inner membrane of the cellular organelle the mitochondria utilize the energy of captured electrons to generate a gradient of H^+ which in turn drives the synthesis of the phosphorylation of ADP to ATP. In the next section, we generically address the origin of these electrons and the H^+ which drive ATP formation.

Molecules that Capture and Transfer Electrons: NADH and FADH2 Formation

NADH and $FADH_2$ are produced during glycolysis, β-oxidation, and other catabolic processes. For the purposes of this review, we will limit ourselves to the process of glycolysis and β -oxidation (Krebs cycle) which takes place in the matrix of the mitochondria.

Glycolysis is one of the earliest evolutionary metabolic pathways. It existed before oxygen was abundant, and therefore even now it occurs anaerobically in the cytoplasm of cells (in the absence of O_2). Glycolysis is the metabolic pathway that converts *glucose* ($C_6H_{12}O_6$) into *pyruvate* ($CH_3COCOO^- + H^+$) (Fig. 8.2), and in the process, four ATP molecules are formed, as well as a total of two molecules of NADH+H^+ are produced [4]. However, because two molecules of ATP are utilized in the beginning of the glycolysis pathway, the net amount of ATP molecules is only two. Thus, the glycolytic pathway is relatively inefficient in generating large quantities of ATP. In the absence of O_2, NADH is utilized in the formation of lactate (from pyruvate), but in the presence of O_2, it may be oxidized by the mitochondria and contribute to generating the H^+ gradient by the transfer of high-energy electrons.

β-oxidation In the presence of O_2, pyruvate (aerobic conditions) enters the mitochondrial matrix (Fig. 8.1) and is converted to acetyl-CoA by the enzyme pyruvate dehydrogenase, initiating the tricarboxylic acid cycle (TCA) or Krebs cycle (see Fig. 8.3) [5]. For every glucose molecule that enters glycolysis, two pyruvate molecules are produced, and therefore, if you are calculating the ATP from this figure, multiply it by two. Starting at the point where pyruvate is converted to Acetyl-CoA, four molecules of NADH are formed from NAD^+, one $FADH_2$ from FAD, and one molecule of ATP from ADP. The other ATP molecules are generated by the electron transport chain (ETC) which is found on the inner mitochondrial membrane.

The ECT and the generation of ATP The inner membrane of the mitochondria (Fig. 8.1) contains many sets of four protein complexes (called protein complexes because each complex contains a number protein subunits), viz., complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome 3 reductase), and complex IV (cytochrome 3 oxidase). Two other factors are involved: Coenzyme Q10, which is associated with complex II, and the cytochrome c complex. These complexes are closely related to each other and are essentially used as a molecular conveyer belt for electrons released during the oxidation of NADH/FADH2 (see the reactions below in Fig. 8.4).

NADH is oxidized by complex I donating two electrons which then is passed to Coenzyme Q, complex III cytochrome c, and then to complex IV. At complex IV, the electrons are finally donated to 1/2 O_2 to form H_2O. The purpose of the electrons conducted from one complex to the next is to provide the energy for the active pumping of H^+ from the mitochondrial matrix across to the intermembrane space.

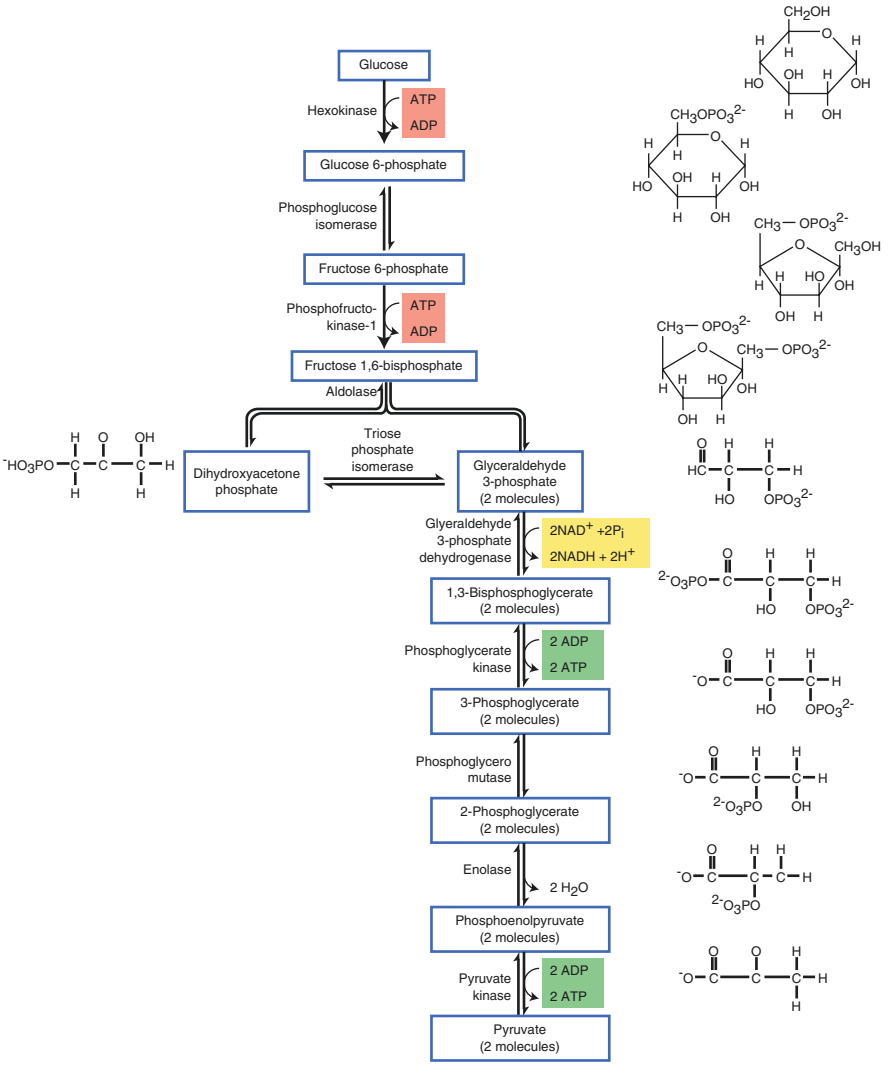


Fig. 8.2 Glycolysis, the metabolic splitting of the molecule glucose, capturing the chemical bond energy in the formation of two ATP (net) and two NADH+2H$^+$ molecules. This is an anaerobic (without O_2) series of reactions, crucial for sperm transit during low O_2 conditions, providing the mitochondria with its metabolic substrate, pyruvate or lactate

At complex I, 4H$^+$ are pumped across, as the electrons reach complex III, 4H$^+$ are pumped across, and as they reach Complex IV, 2H$^+$ are pumped across: so for each NADH molecule oxidized, 10 H$^+$ are pumped into the Intermembrane space. $FADH_2$ is oxidized at complex II and also donates 2e$^-$ to the electron chain, but because it only enters at complex II, only 6H$^+$ are pumped across to the inter membrane space, 4 at complex III, and 2 at complex IV (see Fig. 8.5).

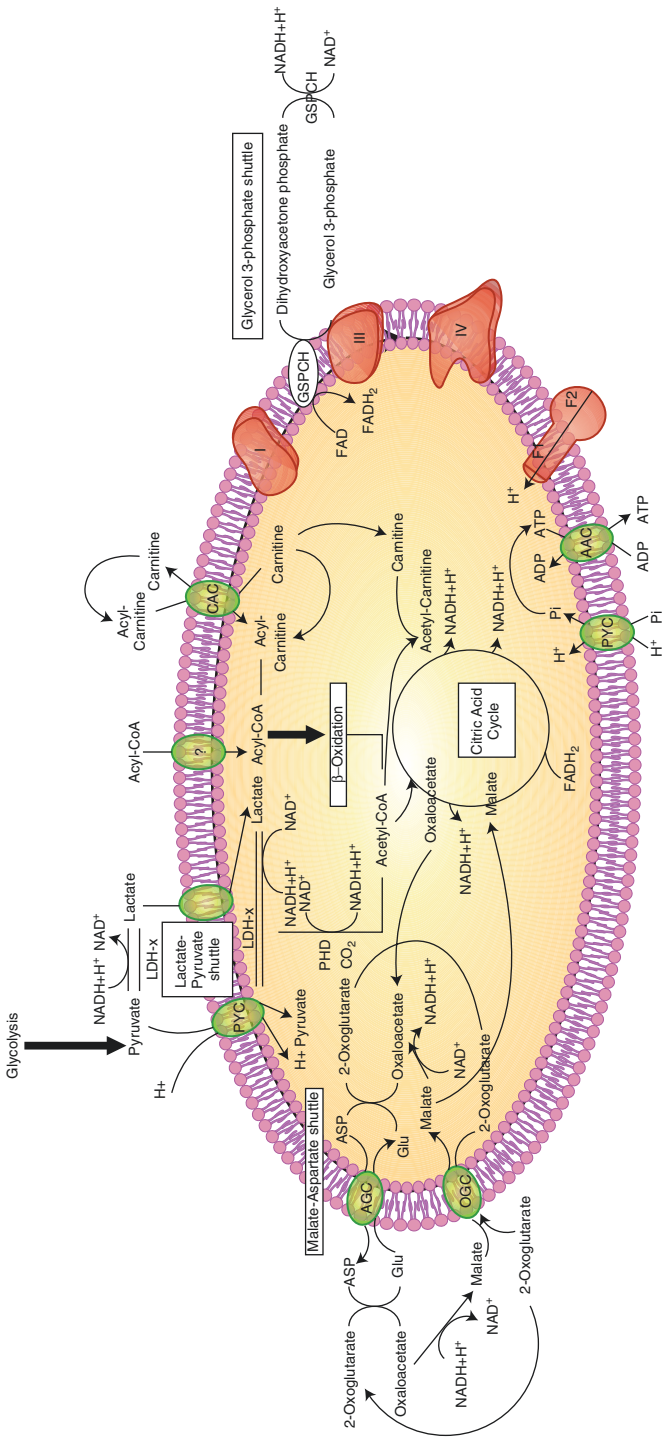


Fig. 8.3 The Krebs/tricarboxylic acid/citric acid cycle are all synonyms for the metabolic cycle which takes place within the sperm mitochondrial matrix. The end product of glycolysis, pyruvate, is metabolized resulting in the formation of high-energy molecules, NADH and $FADH_2$. The molecular complexes (I–IV) and ATP synthase are presented as part of the electron transport chain (ETC). The blue outline represents the inner mitochondrial membrane, with inner space representing the matrix. *AAC* ADP/ATP carrier, *AGC* aspartate/glutamate carrier, *CAC* carnitine carrier, *c* cytochrome c, *G3PDH* glycerol 3-phosphate dehydrogenase (the figure shows the isoenzyme soluble in the cytosol and the isoenzyme integrated in the inner mitochondrial membrane), *LDH-X* isoenzyme X of lactate dehydrogenase (cytosolic and mitochondrial isoenzymes), *OGC* malate/2-oxoglutarate carrier, *PDH* pyruvate dehydrogenase complex, *PtC* phosphate carrier, *PYC* pyruvate carrier, *Q* ubiquinone

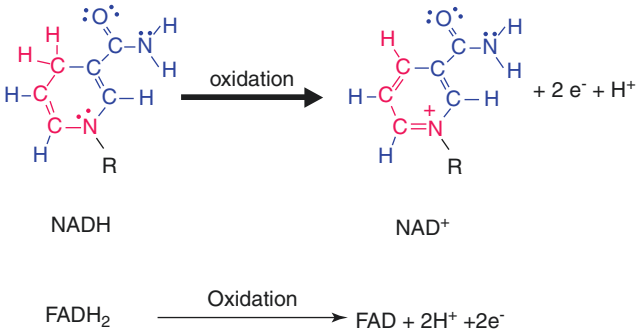


Fig. 8.4 The oxidation of NADH and $FADH_2$ releases electrons to the ETC molecular complexes. The process of oxidation involves a molecule losing electrons. The energy released in this process drives the transport of H^+ through the complexes on the inner mitochondrial membrane, creating a chemiosmotic gradient for the formation of ATP

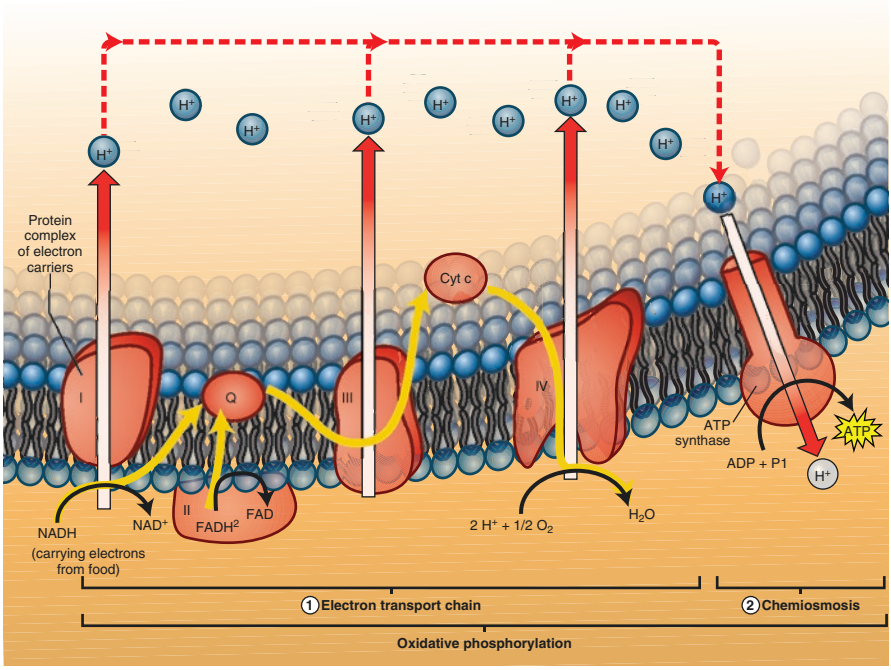


Fig. 8.5 The *electron transport chain* uses high-energy electrons to drive H^+ into the inter membrane space of the mitochondria. Without O_2 as an electron acceptor in the formation of H_2O, β-*oxidation* would grind to a stop. *Chemiosmosis* is the process whereby energy from the H^+ movement down the concentration gradient through the molecule ATP synthase is used to generate ATP

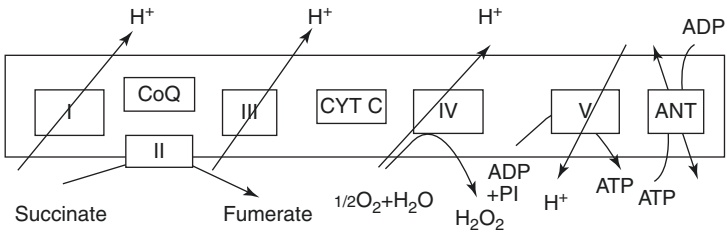
Synthesis of ATP The final part of the oxidation-electron chain saga describes how the intermembrane gradient of H^+ is utilized for ATP synthesis. All these H^+ ions pumped across into the intermembrane space create a chemiosmotic gradient between the inner membrane space and the matrix of the mitochondria. A protein complex on the inner membrane, called ATP synthetase, uses the gradient of H^+ to drive the synthesis of ATP. For every $4H^+$ that flow through the ATP synthetase complex, a single molecule of ATP is synthesized. Thus, for every two molecules of pyruvate that enter the Kreb cycle results in eight NAPH (each producing 3 ATP molecules) and two $FADH_2$ molecules (each producing 2 ATP molecules) being oxidized, generating a total of 28 ATP molecules (see Fig. 8.5).

The H^+ ions are responsible for the development of the mitochondrial membrane potential (MMP) and is often used as an indicator of mitochondrial function. A low MMP would indicate a low concentration of H^+ in the inter membrane space, which in turn indicates that gradient driving ATP synthesis is suppressed. Also, if the mitochondrial membranes become permeable to H^+, it would result in the dissipation of the H^+ gradient and the driving force for the synthesis of ATP. Thus, MMP is often used as an invaluable experimental tool for the analysis of sperm function.

The impact of malfunction of these OxPhos mitochondrial complexes on sperm function: Studies have shown that sperm motility relies heavily on OxPhos. The use of specific inhibitors of the various complexes of the ETC has resulted in specifically understanding the effects of mutations or defective functioning of the various complexes of OxPhos, ROS production, and ATP synthesis. In studies on stallion sperm, inhibiting complex I of the electron transport chain resulted in both decrease motility and membrane integrity which was related to increased hydrogen peroxide (H_2O_2) production and reduced ATP synthesis [6]. Using inhibitors which specifically act on components of complexes I (rotenone sensitive) and III (myxothiazol and antimycin A sensitive) of human sperm mitochondria resulted in an increased production of H_2O_2 (Fig. 8.6) [7]. These studies also showed that inhibiting complex III resulted in increased H_2O_2 production in the intermembrane space. This allowed for easy diffusion of H_2O_2 across the outer membrane of the mitochondria leading to its dissipation into the cytoplasm: no damage to cellular organelle was reported in this case. However, compromising the function of complex 1 (rotenone) led to the increased production of H_2O_2 in the matrix of the mitochondria resulting in peroxidative damage to the midpiece and a loss of sperm motility, which could be reversed by the co-treatment with the antioxidant, α-tocopherol [8]. Thus, understanding the processes of OxPhos is critical to understanding the basis for sperm-based infertility and the clinical intervention that is appropriate.

Mitochondrial DNA and Nucleic DNA

Mitochondria have their own DNA (mt-DNA), mitochondrial ribosomes, and their own machinery for protein synthesis within the mitochondrial matrix. mt-DNA is circular and structured like a plasmid where mt-DNA are constructed out of two circular



Complex	I	II	III	IV	V
Inhibitor	Rotenone		Antimycin	KCN	Oligomycin
nDNA subunits	>18	4	8	10	10
mtDNA subunits	7	0	1	3	2

Fig. 8.6 The electron transfer chain. Protons (H^+) are pumped into the surrounding media and establish an electrochemical gradient which provides the energy for ATP synthesis. The electrons passed down the ETC, combine with the protons, and react with molecular oxygen resulting in the production of H_2O. Most of the subunits for the complexes are encoded by nDNA, but all of the complexes except for complex II have one or more of their subunits encoded by mt-DNA. *CoQ* Coenzyme Q, *CYTC* cytochrome c, *ANT* adenine nucleotide transporter, *nDNA* nuclear DNA, *tDNA* mitochondrial DNA, *KCN* potassium cyanide

strands, an outer heavy strand and an inner light strand. Human mt-DNA codes for 16,569 base pairs and 37 genes, 13 of which are involved in the synthesis of ATP. These genes generally provide instructions for the synthesis of parts of the enzymes (13 polypeptide components) which are specifically involved in OxPhos as well as the structures involved in the molecular machinery of transcription and translation (protein synthesis), viz., 2 ribosomal RNAs and 22 transfer RNAs. However, a large number of essential protein components are specifically encoded for by the nuclear DNA and transported into the mitochondria. It is estimated that some 250–300 nucleus-encoded proteins are dedicated and essential to mitochondrial gene expression (see Fig. 8.5).

Owing to the dual genetic origin of the protein subunits of OxPhos, a high degree of coordination is required to ensure the synthesis, assembly, and the insertion of the complex protein subunits and ATP synthetase of the inner membrane ETC complexes. Recently, contrary to a current thinking which suggests that mitochondrial DNA is under the control of nuclear DNA, new studies have shown that under metabolic stress, a peptide (MOTS-c), encoded by the mt-DNA, can relocate to the nucleus, bind to chromatin, and regulate nuclear gene expression to respond adaptively to homeostatically reverse the metabolic stress [9]. These findings suggest that the mitochondrial and nuclear genomes co-evolved and that they have the ability to regulate each other in a genetically integrated manner.

Furthermore, mitochondria do not have the complete DNA machinery to repair ROS-related DNA breaks, cross-links, and modified bases as do the nucleic genome, and thus, mt-DNA damage by exogenous and endogenous ROS sources may lead to mutations that are unrepairable. Therefore, exogenous or endogenous sources of ROS that impact the mt-DNA may be crucial in understanding male infertility.

The Fate of mt-DNA

Nuclear material is inherited from both sexes, but mitochondrial DNA is almost exclusively inherited maternally. This uniparental mt-DNA inheritance is a process which is conserved in humans, mammals, and most eukaryotic organisms. Paternal mitochondria have a specific role in supplying the energy for sperm motility and any other metabolic process requiring energy for sperm viability and its fertility. The importance of mitochondrial persistence in the sperm, while most other organelles are purposefully eliminated, is to ensure that sperm motility is as efficient as possible. Nevertheless, this also exposes the sperm while developing and in transit, to possible oxidative stress (OS) damage.

Once the sperm has entered into the cytoplasm of the ovum, both the mitochondria and the nucleus are still present; however, while the sperm nucleus persists, the paternal mitochondria are eliminated. There are still no clear reasons for the elimination of the paternal mitochondria (and its genome) other than mitigating against the OS (or any other transit damage) that may have occurred via ATP production. An alternate rationale suggests that maternal uniparental mt-DNA inheritance may protect against conflicts occurring between two sets of differing yet "normal" DNA haplotypes, during the inheritance of biparental mt-DNA. Sharpley et al. [10] showed that heteroplasmic mt-DNA mice presented significant physiological, cognitive, and behavioral deficiencies compared to homoplasmic mt-DNA mice. Thus, inheriting different sets of mt-DNA haplotypes may compromise OxPhos efficiency, presenting in a variety of symptomatic impairments.

Nevertheless, although it is relatively well established and accepted that paternal mt-DNA is eliminated, both paternally during spermatogenesis and after fertilization, a recent study showed that paternal could be inherited under certain conditions and have provided evidence for the persistence of paternal mt-DNA in offspring [11]. Although human maternal inheritance of mitochondrial DNA remains the norm, one cannot exclude paternal-mitochondrial inheritance as it is a persistent anomaly that remains to be clarified in future research.

Effects of mt-DNA Mutations on Fertility

Mitochondrial DNA is far more prone to mutations than nucleic DNA because mt-DNA are in the immediate vicinity (in the mitochondrial matrix) to where ROS is generated during OxPhos. Mutations to mt-DNA almost always result in disturbances to the molecular subunits of the ETC which could seriously impact the production of ATP and, consequently, sperm motility.

As recent as 1993, mt-DNA mutations were correlated with male infertility when Folgerro et al. reported reduced sperm motility in individuals who have structural defects in their mitochondria. This was shortly followed by a report of a man presenting with spermatogenic failure as a result of mitochondrial 4977bp

deletion [12]. In 1997, the impact of mt-DNA mutations was further entrenched by a report of an oligoasthenozoospermia individual who had mt-DNA rearrangements [13]. For a historical review of the effects of mutations, see Rajender et al. [14].

It is clear, therefore, that mutations of mt-DNA, whether as a point or serial mutation, have the potential to seriously compromise spermatogenesis, motility, and the fertility of sperm.

Metabolic Substrates for Sperm Mitochondria

Since the 1940s, scientists have been investigating the role various metabolic substrates have on the functioning of the mitochondria of sperm. Early experiments often yielded different and also conflicting results. These early experiments mostly used intact motile sperm and measured oxygen consumption polarographically. In these experiments, the addition of substrates (e.g., pyruvate, malate, fumarate, citrate) that would be used exclusively to drive mitochondrial-based respiration had little or no effects over baseline experimental data. These experiments led scientist to believe that OxPhos and the mitochondria had limited effect on sperm function and that in effect the dominant metabolic pathway was glycolysis. However, this was contrary to the structural design of the sperm which shed most organelle with the exception of the acrosome (which emanates from the Golgi apparatus) and the rearranged mitochondria found conspicuously in the midpiece of sperm. Secondly, glycolysis is a very inefficient pathway in terms of ATP production providing only two ATPs per glucose molecule metabolized. A new experimental method was devised which involved incubating sperm in a hypotonic buffer solution which made the sperm plasma membrane more permeable while leaving the mitochondrial membranes unaffected. This allowed free access to exogenously added substrates. Using this method, Ferramosca et al. [15] showed that human sperm mitochondria utilized both pyruvate and malate efficiently for respiration. Furthermore, this method allowed for the use of various OxPhos inhibitors to further elucidate the functioning of sperm-based mitochondrial respiration. These experiments also illustrated the importance of OxPhos as an efficient producer of ATP and thus to sperm functionality. It is, however, important to recognize that glycolysis is an important energy-producing pathway especially under low-oxygen conditions and that secondly the glycolysis pathway is important in providing the mitochondria with carbon substrates for complete oxidation.

Ferramosca et al. [15] showed that sperm mitochondria could also internalize and utilize both lactate, palmitoyl-CoA, and malate as substrates for OxPhos. This supports the rationale that sperm requires a variety of energy processes at its disposal while traversing the female reproductive tract to the oocyte and that the ability of the mitochondria to utilize several different substrates is also expedient to the sperm's fertilization capacity.

Mitochondrial Membrane Potential

As indicated, the functionality of the mitochondria is determined by the mitochondrial membrane potential (MMP), which plays a fundamental role not only in the induction of apoptosis [16, 17] but also in the maintenance of the ATP production [18] and sperm motility, quality, and fertilization success in in vitro fertilization (IVF) [19–23]. On the other hand, although data by Sharbatoghli et al. [24] confirm the significant association of MMP with motility, no correlation of MMP with fertilization data such as fertilization rate, embryo quality, cleavage, and pregnancy rate was found after intracytoplasmic sperm injection (ICSI). This implies that MMP correlates with motility and does not play a role for ICSI, where all physiological barriers for sperm entry into the oocyte are bypassed. This is in contrast to IVF, where spermatozoa have to be motile in order to fertilize the oocyte.

Latest research shows that the sperm MMP in fact regulates motility as patients with poor sperm motility had lower MMP than fertile healthy men [25]. In the same study, the authors showed that oxidative uncoupling of the mitochondrial ETC not only reduced the MMP but also motility. This relationship could be mediated by the uncontrolled release of reactive oxygen species (ROS) from the uncoupled ETC, which in turn damages cellular functions including motility and is causing nuclear DNA fragmentation [26–28]. Even other sperm functions such as acrosin activity and acrosome reaction are dependent on proper mitochondrial function, which reflects in an undisturbed MMP [29].

Mitochondrial Function in Swim-Up Sperm

Considering that the mitochondria produce the necessary energy in form of ATP for all physiological processes including motility, this close relationship between MMP and motility is obvious. Swim-up sperm have been shown to have the highest motility and significantly higher MMP as compared to sperm in the pellet [25]. Hence, MMP could be a useful and sensitive test in the diagnostic setup for male infertility [30].

Apoptosis: Site for Intrinsic Apoptotic Pathway

Apoptosis, the programmed cell death, is well known in somatic cells to eliminate dysfunctional cells. Although different pathways for the activation of this process have been postulated, stronger evidence in vertebrates is only available for pathways, the death receptor pathway (extrinsic pathway), and the mitochondrial pathway (intrinsic pathway) [31, 32]. While the extrinsic pathway is triggered by plasma membrane-bound death receptors of the tumor necrosis family such as Fas, which trigger, among

others, caspase-8 to activate executing caspase-3, in the intrinsic pathway, mitochondria are playing a major role [17]. However, mammalian sperm are normally prevented from entering this pathway because of their phosphatidylinositol 3-kinase (PI3K) activity [33]. Only if PI3K is inhibited, sperm enter the intrinsic apoptotic pathway leading to cytosolic caspase activation, excessive mitochondrial ROS production followed by loss of motility, cytoplasmic vacuolization, and oxidative DNA damage.

According to Aitken et al. [34], sperm and normal somatic cells differ in two major points for apoptosis: (i) a structural difference in the cells as in sperm cytoplasm and mitochondria are compartmentalized in the midpiece of the male germ cells and are thus clearly structurally separated from the nucleus with the DNA; (ii) in contrast to somatic cells, sperm exhibit a significantly shortened base excision repair (BER) pathway with only one enzyme, 8-oxoguanine DNA glycosylase (OGG1), being present. This enzyme is located in the nucleus and mitochondria and can remove 8-hydroxy-2′-deoxyguanosine (8OHdG) from the cell and release the base adduct into the extracellular space. Downstream components of this pathway like apurinic endonuclease-1 (APE1) are missing, resulting in the formation of abasic sites that have already been affected by 8OHdG and eventually leading to DNA strand breaks. This is a mechanism of DNA damage that has been described to trigger cancer in other cells. Consequently, these DNA base changes are not only mutagenic but contribute significantly to DNA damage [35].

Another consequence of sperm not having APE1 in the intrinsic pathway of apoptosis is that sperm are not able to produce 3’-OH termini that are required by terminal deoxyribonucleotidyl transferase to label the sticky ends in the TUNEL assay. Consequently, the TUNEL cannot detect these early DNA damages but only at a later stage when the sperm cells are close to death and a DNase is activated [36].

Levels of ROS

The sperm DNA damage can be caused via two mechanisms, namely, oxidative stress and DNA cleavage mediated by endonucleases, both of which are involved if sperm are undergoing apoptosis [33]. Although mitochondria normally produce a certain limited amount of ROS, this mitochondrial ROS production can be boosted by various factors including oxidative stress, cryopreservation [37, 38], lack of antioxidant protection [39, 40], exposure to oxidants and radiation [41, 42], and seminal excessive amounts of leukocytes [43–45]. An early sign of the initiation of the apoptotic pathway is an increase in the mitochondrial ROS production, which leads to a loss in sperm motility [33]. Furthermore, these ROS also trigger damage to the plasma membrane as they initiate the process of lipid peroxidation and the generation of cytotoxic aldehydes such as 4-hydroxynonenal and acrolein [46]. Subsequently, these processes will lead to a breakdown of the mitochondrial membrane potential with electron leakage at the electron transfer chain generating superoxide which dismutates to hydrogen peroxide. The latter, in turn, triggers more lipid peroxidation with more mitochondrial ROS leakage leading into a vicious cycle

[26] and eventually these sperm cells eventually entering the intrinsic apoptotic pathway with externalization of phosphatidylserine as another early marker of apoptosis and eventually sperm nuclear DNA damage as late sign of apoptosis [47].

Effects of Temperature on Mitochondrial Function

Considering that testicular temperature is carefully regulated at 35°C and vaginal and uterine temperatures are at 37°C, it is expedient to ask if temperature has any effects on sperm and its mitochondrial function, and if so, what? It is already well established that cryptorchidism (undescended testis) if untreated and left in the abdominal cavity will result in infertility. Approximately 10% of infertile men have a history of cryptorchidism and orchidopexy (surgical assistance to descend the testis) [48]. Increased testicular temperature results in increased apoptosis and has a negative effect on pachytene spermatocytes, early spermatids, and even the supporting Sertoli cells of the germinal epithelium but has no effect on testicular interstitial Leydig cells [49]. In experiments in rats, in which one testis was surgically positioned in the abdomen for a period of 4 weeks (essentially a 2-degree increase in testicular temperature), ejaculatory capacity was decreased by more than 25%. In human experiments, in which one group of subjects was exposed to 43°C baths for 30 min for 10 consecutive days, while another group was exposed every third day, the increased temperature effected the sperm mitochondria of both groups by an increased proportion of disrupted MMP [50]. This indicates that increased testicular temperature suppresses OxPhos decreasing the proton gradient in the intermembrane mitochondrial space which in turn drives ATP production.

In recent times, ejaculates may be frozen, and some may be prepared for assisted reproductive techniques, where they are sometimes kept at room temperature and at other times at 37°C. So just how will these environmental temperature changes affect post-ejaculated sperm and their mitochondria? Studies have suggested that increasing the temperature of post-ejaculate could lead to the suppression of sperm mitochondrial OxPhos, by decreasing the activity of the respiratory chain complexes (I and IV), decreasing the active transport of protons into the intermembrane space of the mitochondria, and thereby decreasing the driving force for the production of ATP [51]. The lower ATP availability is directly implicated for the heat-stress-induced decrease in sperm progressive motility. This suggests that sperm manipulations should be performed at room temperature rather than at 37°C.

Concluding Remarks

Mitochondria are one of the few cellular organelles that are not eliminated during the process of spermatogenesis in sperm. They are conspicuously packaged into the midpiece of the male germ cell and, therefore, intuitively by design, are crucial to

the function of sperm motility and by implication male fertility. Sperm mitochondria are able to utilize the glycolysis metabolic end products, pyruvate and/or lactate, as well as other substrates to generate ATP molecules to ensure the essential functions of sperm during transit, viz., motility, hyperactivation, and the acrosome reaction at the ovum. A malfunction of the mt-DNA, or nucleic-DNA coding for ETC molecular components, has a restrictive effect on sperm motility and hence should be scrutinized in assessing male infertility. Routine assessment of MMP as a functional sperm parameter will enable clinicians to cursorily evaluate sperm mitochondrial OxPhos function and, therefore, the ability of sperm to generate ATP available for sperm function. It is also clear that sperm mitochondria are vulnerable to OS, which has been shown to compromise sperm function. As post-ejaculated sperm are routinely used in assisted fertility procedures, current research suggests that if sperm are held at room temperature, improved mitochondrial function may improve fertilization success. Optimal mitochondrial function is both central and crucial to male fertility, and therefore, understanding and monitoring of its function is expedient in treating male infertility.

Review Criteria

An extensive search of studies examining mitochondrial function and dysfunction and its impact on male infertility was performed using PubMed. The searches were not limited for time. Yet, the most recent records were preferred. The overall strategy for study identification and data extraction was based on the following keywords: “male infertility,” “mitochondrial function,” “mitochondrial dysfunction,” “mitochondrial DNA,” “mt-DNA,” “nuclear DNA,” “mitochondrial membrane potential,” “reactive oxygen species,” and “ROS” as well as the names of most prominent researchers working on this topic. Articles published in languages other than English and data that were solely published in conference or meeting proceedings or websites were not included. Websites and book chapter citations provide conceptual content only.

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Chapter 9
Sperm DNA Fragmentation and Male Infertility

Manesh Kumar Panner Selvam, Pallav Sengupta, and Ashok Agarwal

Key Points
- Chromatin organization and protamination state determine the sperm DNA integrity.
- Molecular changes due to sperm DNA damage are reflected as alterations in the sperm proteome.
- SDF adversely affects the fertilization rate and embryo development.
- SDF testing is highly recommended in men with idiopathic infertility.

Introduction

Worldwide concern regarding upsurge of male infertility contributing to almost 50% of the overall infertility cases urges specific research interventions to address its potential causes [1]. Considerable advent of assisted reproductive technology (ART) could hardly mitigate stillbirth complications [2]. Proper approach to ameliorate male fertility should not be compensated with ART. Management of male infertility, which in most of the cases remains idiopathic, can be effective once its diagnosis is feasible. In order to do so, the etiology of male infertility from every

M. K. Panner Selvam · A. Agarwal (✉)
American Center for Reproductive Medicine, Cleveland Clinic, Cleveland, OH, USA
e-mail: agarwaa@ccf.org

P. Sengupta
Department of Physiology, Faculty of Medicine, Bioscience and Nursing, MAHSA University, Jenjarom, Selangor, Malaysia

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aspect should be considered and possible mechanisms be explored and conceptualized. In this regard, understanding the molecular and genetic processes associated with sperm functions is of prime importance. The significance of sperm DNA integrity in association with sperm function tests is regaining research priority, which has huge impact on reproductive outcomes. Sperm DNA fragmentation (SDF) owing to various exogenous and endogenous factors directly affects sperm functional and morphological characteristics, rendering them impotent in carrying out reproductive functions [3, 4]. SDF assays help in advancing clinical andrology by several steps by offering a potential diagnostic tool for male infertility. SDF testing, though not yet recommended for routine testing in the evaluation of infertile men, is being acknowledged in the American Urological Association (AUA) and European Association of Urology (EAU) guidelines [5, 6].

This chapter elucidates the etiology of SDF with its contribution to male infertility, the mechanisms by which environmental, lifestyle, and endogenous factors mediate SDF, and the contemporary SDF assessments in the diagnosis of male infertility.

Etiology of Sperm DNA Damage

Sperm structure is precisely made for the successful transmission of the haploid genome to the secondary oocyte. The success of fusion and delivery of DNA content is directly related to the compaction of genetic material in an extremely limited volume of the nucleus. Mammalian sperm chromatin differs from somatic cells in structure and composition, which maintains genetic integrity during transport of the paternal genome into oocyte [7]. Protamination is a unique process that replaces histones with positively charged protamines during the nuclear chromatin condensation process. Defect at any stage of this process may result in SDF during its transport and fertilization. Indeed, the extent of DNA damage/breaks varies from sperm to sperm even in fertile men [8]. Emerging evidences support the significance of chromatin organization during fertilization and embryo development [9–11]. However, in normal state, meiotic prophase passes through recombination checkpoint that restricts progression to meiotic division-I till the DNA is completely repaired or the incompetent impaired spermatocytes are removed [12]. Ligation of DNA breaks is crucial for both conserving the primary DNA integrity and reassembly of the DNA loop domain for the genome expression [13]. This reassembly includes delicate steps such as chromatin loosening through histone hyperacetylation by endogenous nuclease activity and ligation of DNA breaks by topoisomerase II [14]. Usually chromatin packaging around the new protamine cores and restoration of DNA integrity are accomplished during epididymal transit [15]. However, the presence of endogenous nicks in spermatozoa after epididymal transit may indicate an improper chromatin packaging at spermiogenesis and an incomplete maturation process. The differential susceptibility of chromosomes to sperm DNA fragmentation is determined based on its association with either of the DNA packaging molecules such as histones or protamines [16].

Besides the defects in sperm chromatin compaction, numerous other intrinsic and extrinsic factors have been reported in the etiopathogenesis of SDF, including varicocele, infection, advanced male age, heat stress, lifestyle factors, environmental toxins, and ionizing/non-ionizing radiations [17, 18]. Most of these etiologies are mediated by reactive oxygen species (ROS) leading to elevated SDF [19]. Abortive apoptosis [20] and defective maturation [21] correlate with the role of intrinsic factors in testicular SDF. Moreover, evidence show more DNA fragmentation in epididymal and ejaculated sperm than testicular sperm, signifying the impact of extrinsic factors [22]. Presence of large amount of polyunsaturated fatty acids (PUFA) in the plasma membrane makes sperm susceptible to ROS-induced damage [23].

The close relationship between ROS and SDF is also evident from the etiopathologies of all grades of clinical varicocele. The imbalance between ROS (produced by testicular hypoxia, scrotal hyperthermia, reflux of metabolites, and endocrine disruption) and protective antioxidant system was demonstrated by the higher level of ROS and lipid peroxidation products in infertile men with varicocele than infertile men without varicocele [24]. Moreover, treatment of varicocele is effective in decreasing both ROS [25] and SDF [26].

Thus, it is apparent that sperm functions and morphology are impaired via multifarious intrinsic and extrinsic factors. The abnormal spermatozoa together with these factors lead to increased ROS levels that afflict sperm DNA integrity and thereby results in infertility, impaired ART outcomes, and birth defects, as illustrated in Fig. 9.1.

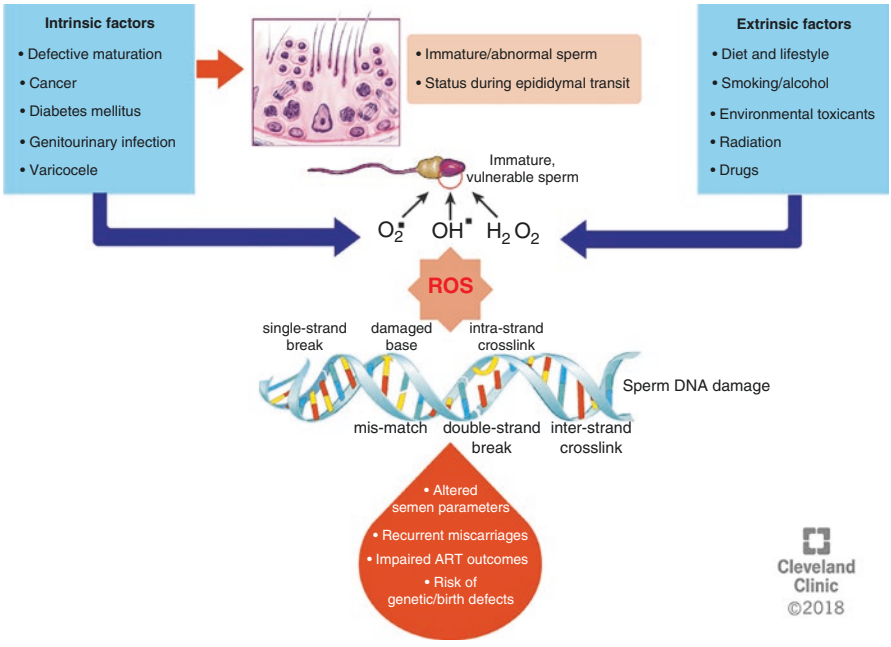


Fig. 9.1 Reactive oxygen species produced by intrinsic and extric factors and its impact on the sperm DNA to disrupt its integrity affecting the reproductive outcomes. (From Cleveland Clinic Foundation; with permission)

Molecular Changes associated with Sperm DNA Fragmentation

Sperm DNA damage affects both the nuclear and mitochondrial genome, as well as the molecular machinery at the subcellular level [17, 27, 28]. SDF also causes alterations in the sperm ultrastructure, such as vacuolation in the nucleus, severe sperm morphological abnormalities including teratozoospermia [29]. These changes adversely affect normal sperm functions such as hyperactivation, capacitation, and acrosome reaction which are critical for the binding of spermatozoa with the oocyte during fertilization [30, 31]. Especially, the proteome of the sperm and seminal plasma are altered in the patients with high SDF [28, 32]. It has significant impact on sperm protein expression and molecular processes associated with triacylglycerol metabolism, energy production, protein folding, response to unfolded proteins, and cellular detoxification [28]. Also, the postgenomic pathways associated with sperm metabolism, function, and protection against oxidative stress get affected in spermatozoa with high DNA fragmentation [28]. Elevated SDF also disrupts spermatogenesis by altering the expression of prolactin-induced protein and its precursor protein (pPIP). Most of the proteins associated with DNA binding (such as sperm protein associated with nucleus in the X chromosome and histone proteins), oxidative stress, and mitochondrial function are differentially expressed [33].

Seminal plasma proteome also reflects the pathology associated with SDF, and these are modulated depending upon the extent of sperm DNA damage [32]. Intasqui et al. also reported that the postgenomic pathways are altered in the seminal plasma of normozoospermic men with low and high DNA fragmentation. Molecular pathways such as fatty acid binding and prostaglandin biosynthesis functions were reported to be enriched in DNA-damaged spermatozoa [34]. Cysteine-rich secretory protein LCCL domain-containing 1 (CRISPLD1), cysteine-rich secretory protein LCCL domain-containing 2 (CRISPLD2), and retinoic acid receptor responder protein 1 were proposed as biomarkers for low SDF, whereas proteasome subunit alpha type-5 protein was considered to be potential seminal biomarker for high SDF [34]. The molecular changes in the seminal plasma of smokers with high SDF were mainly related to decreased acrosome integrity and mitochondrial activity. Furthermore, the seminal plasma of patients with high SDF portrays activation of the pathways associated with positive regulation of prostaglandin secretion, protein kinase A signaling, cytokine mediated signaling, and acute inflammatory responses [35]. In infertile patients exhibiting high levels of ROS along with SDF, enzymes linked to DNA binding mechanism were altered in the seminal plasma [36].

Overall, the molecular protein signatures of both the spermatozoa and seminal plasma are altered in high SDF conditions. Differentially expressed proteins may serve as potential biomarkers in the sperm pathology with compromised DNA integrity.

Male Infertility Factors/Conditions Associated with SDF

Studies reporting the link between male factor infertility and SDF have diverse observations. Many studies have correlated SDF and male infertility with evidence of decreased sperm functions [37–39], while others have reported that high SDF can also be observed in sperm with normal motility and morphology [40–42]. An elevated level of SDF is also reported in men with abnormal semen parameters and normozoospermic partners of infertile couples [43]. However, SDF is a crucial factor to maintain male fertility and development of a healthy embryo. In a recent article by Agarwal et al., the role of female factors in the management of SDF for a better outcome in ART has been elucidated [44]. Authors discussed the complex interplay between the SDF and ovarian reserve on the clinical outcomes of ART; the presence of an intact oocyte repair machinery in good quality oocytes has a pivotal role in reproductive outcomes including SDF which serves as a safety check to avoid passage of defective genetic information to offspring [44]. However, several male infertility factors are associated with SDF.

Male Age

It has been reported that among the couples seeking treatment by ART, fathers are significantly older compared with those not needing ART (36.6 vs. 33.5 years) [45]. Men with age of 40 years or older are also found to be at higher risk of sperm DNA damage [46]. But some of the studies have reported no correlation between paternal age and SDF [47, 48]. However, most of the studies have reported that with increasing male age, the incidence ROS generation [46] and diploidy/aneuploidy increases in sperm [48, 49].

Diet, Lifestyle, and Modifiable Risk Factors

The correlation between oxidative DNA damage and the consumption of foods supplemented with antioxidant compounds, with better general and reproductive health, has been reported in diverse studies [50, 51]. Most of the reports indicate that increased intake of individual antioxidants or antioxidant-rich foods can reduce the basal level of sperm DNA damage [52, 53]. It is apparent that endogenous sperm DNA oxidation levels are modulated through diet or supplementation, but a number of variables such as type and dose of antioxidant, basal level of antioxidant plasma concentrations, and smoking or alcohol consumption can interfere with the effectiveness of the outcome. Smoking [54] and alcohol consumption [55] trigger SDF separately as well as in combination [56]. The mechanism of smoking or alcohol-mediated SDF is due to the excess generation of ROS that affects sperm quality and, ultimately, fertility potential of the spermatozoa. In chronic smokers, activation of

the checkpoint kinase 1 (Chk1) facilitates S and G2 checkpoint arrest, in response to DNA damage. The expression of Chk1 is associated with SDF and apoptosis, the reduction of which may lead to decreased sperm repair and increased sperm apoptosis, with a subsequent effect on semen quality [54]. Reports regarding SDF and alcohol consumption suggest that during intrinsic apoptotic cascade, hydrogen peroxide released from the sperm mitochondria can induce SDF in the nucleus [57]. Much later in the apoptotic process, the sperm DNA begins to fragment [58].

Obesity

There has been an emerging concern over the past few decades on the impact of obesity on male fertility. Infertility has been linked to male overweight or obesity, and conventional semen parameter values alter in case of high body mass index (BMI) [59]. Male obesity is associated with an increased risk of sperm DNA damage and lower sperm motility and thus poor sperm quality [59]. Numerous human and animal studies have determined that a relationship between obesity and reduced sperm DNA integrity exists, despite the use of a variety of different methodologies to measure sperm DNA integrity [60]. Obesity induces OS and disrupts endocrine balance in men that brings about a negative impact on sperm DNA integrity [61].

Environmental Toxicants

Environmental and occupational exposure of heavy metals [62], pesticides [63], and other endocrine disrupting chemicals (EDCs) are involved in deteriorating the male reproductive health resulting in male infertility. Exposure to these EDCs also positively correlates with SDF [64]. Different agents that act on germ cells at various stages of development usually showed SDF when those germ cells arrive in the epididymis or in the ejaculate. Some of these treated samples were capable of successful in vitro fertilization but with frequent embryo failure. Extensive DNA fragmentation probably cannot be repaired by the oocyte, and the spontaneous abortion rate approximately doubles in men with more than 30% of sperm showing DNA fragmentation [65]. DNA fragmentation is an excellent marker for exposure to potential reproductive toxicants and a diagnostic tool for potential male infertility.

Chemo/Radiotherapy

In the last few decades, numerous reports have confirmed negative impact of ionizing and non-ionizing radiations on male infertility [18, 66]. Ionizing radiations from medical equipment and radiotherapy for cancer treatment positively correlate

with SDF and declining sperm quality [18]. Cancer treatments are well known to adversely affect male fertility. Reduction of sperm count arises from the cytotoxic effects of chemo- or radiotherapy upon the spermatogenic epithelium [67]. Studies have also confirmed that radiotherapy in testicular germ cell tumors is associated with an increase in SDF compared to chemotherapy alone [68]. Non-ionizing radiations from cell phones, Wi-Fi, and other radioactive sources also have significant negative impacts on male fertility and sperm DNA integrity [66].

Infections and Testicular Trauma

As discussed above, multiple pathological factors acting at both intratesticular and post-testicular levels may contribute to sperm DNA damage. Bacteriospermia is one of the pathological conditions that manifests as acute or chronic inflammation and increases leukocyte infiltration in the genital tract resulting in higher ROS production [69]. Patients with leukocytospermia, *Chlamydia* and *Mycoplasma* infections, testicular cancer, and varicocele have also reported to have more SDF caused by excessive production of ROS [26, 70, 71]. However, SDF in patients with *Chlamydia* and *Mycoplasma* infections were reported to decrease after a course of antibiotics [70].

Techniques Used for SDF Assessment

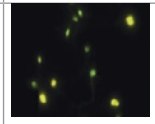
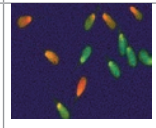
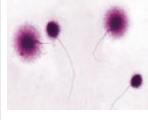
A variety of assays are used to assess sperm DNA damage. These are classified as direct and indirect tests, which either measure the maturity and integrity of sperm chromatin or DNA fragmentation (Table 9.1). Most commonly used SDF tests are sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), sperm chromatin dispersion (SCD), and the Comet assay. A cross-sectional survey across 19 countries by Majzoub et al. showed that 30.6% of SDF measurements are done using TUNEL and SCSA, 20.4% and 6.1% using SCD and single-cell gel electrophoresis (Comet), respectively [72]. The test results of each assay are different and are not interchangeable.

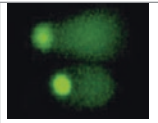
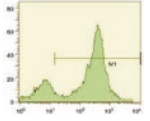
Sperm Chromatin Maturity Testing

Aniline Blue Staining (AB)

Immature spermatozoa contain lysine-rich histones, and mature spermatozoa have arginine and cysteine abundant protamines. AB is an acidic dye that reacts with the lysine and stains the immature spermatozoa blue, whereas matured spermatozoa

Table 9.1 Different techniques to measure sperm DNA fragmentation

Test	Principle	Method	Result		Advantage	Disadvantage
Acridine orange (AO) test	Metachromatic shift in fluorescence of AO when bound to DNA breaks	Acid denaturation, followed by staining by AO Uses fluorescent microscopy	Normal DNA fluoresces green Denatured DNA fluoresces orange-red		Rapid and simple Inexpensive	Interlaboratory variations Lack of reproducibility
Chromomycin A3 (CMA3) staining	Compete with protamine for the same binding site in DNA	Staining by CMA3	Highly positive test reflects a low DNA protamination state associated with poorly packaged sperm chromatin		Strong correlation has been demonstrated with other SDF assays	Inter-observer variability Interlaboratory variability not tested Technically demanding
SCSA	Measures the susceptibility of sperm DNA to denaturation	Acid denaturation, followed by staining by AO Measurement by flow cytometry Uses fluorescent microscopy	Normal DNA fluoresces green Denatured DNA fluoresces orange-red Result presented as DNA fragmentation index (% DFI) and high DNA stain ability (%HDS)		Standardized protocol available Rapid evaluation of large number of spermatozoa Correlations with results of other SDF assays Established clinical thresholds can be performed on fresh or frozen samples	Indirect assay involving acid denaturation Proprietary protocol with no commercial assay Requires expensive instrument and highly skilled technicians
SCD/Halo test	Assess dispersion of DNA fragments after denaturation	Agarose-embedded sperm are subjected to a denaturing solution to remove nuclear proteins Uses fluorescent microscopy to observe chromatin dispersion after staining	Sperm with fragmented DNA do not produce halo Characteristic halo of dispersed DNA loops are observed in sperm with non-fragmented DNA Result presented as percentage of sperm with non-dispersed chromatin		Relatively simple test with commercial kit available	Indirect assay involving acid denaturation Inter-observer variability Time-consuming and labor-intensive if using microscopic evaluation

SCGE/Comet assay	Electrophoretic assessment of DNA fragments of lysed DNA	Gel electrophoresis performed in alkaline or neutral conditions	Size of comet tail represents the amount of DNA fragments that stream out of the sperm head Result presented as mean amount of DNA damage per spermatozoon		Direct assay can be performed on few sperm Detect multiple types of DNA damage of individual spermatozoon Result correlates well with other SDF assays	Requires fresh sample Inter-observer variability Time-consuming Requires experienced observer
TUNEL	Quantifies the enzymatic incorporation of dUTP into DNA breaks as percentage of fluorescent sperm	Labeled nucleotides are added to site of DNA fragmentation Fluorescence is measured by flow cytometry or fluorescence microscopy	Sperm with fragmented DNA showed fluorescence Result presented as percentage of fluorescent sperm		Direct assay can be performed in fresh or frozen samples Can be performed on few sperm Detects both single- and double-strand DNA breaks Commercial assay available Reference sample is not required	Requires standardization among laboratories Time-consuming Immature spermatozoa are not evaluated Variable clinical thresholds reported in the literature

Data from Cho and Agarwal [37]

remain unstained. Stained spermatozoa are visualized under simple bright field microscope. The integrity of the sperm chromatin is assessed based on the intensity of the stain [73].

Chromomycin A_3 (CMA3)

Protamination state of the spermatozoa determines its chromatin integrity status. The lesser the protamine content, the poorer the DNA packaging and the higher the sperm DNA damage. CMA3 binds to the sperm DNA deficient of protamine and stains light yellow [74]. The intensity of color is high in sperm with increased protamination [75]. Fertilization rate in ICSI were reported to be significantly lower with DNA damage of >30% in semen samples determined by CMA3 assay [76].

Sperm DNA Fragmentation Testing

Sperm Chromatin Structure Assay (SCSA)

SCSA is an indirect SDF test and used to detect breaks in the single-stranded DNA (ssDNA) of sperm. Acridine orange (AO) dye binds with the ssDNA and emits red fluorescence, whereas AO bound to double-stranded DNA emits green fluorescence, and the signals are captured using a flow cytometer [77]. SCSA can be done on both fresh and frozen sperm, and a clinical reference value for DNA fragmentation index (DFI) of 30% was established for SCSA [78, 79].

Sperm Chromatin Dispersion (SCD) Test

SCD is also known as halo assay and was first introduced by Fernández et al. [80, 81]. The sperm cells embedded into the low-melting agarose-coated slides produce halos/chromatin dispersion when denatured with acid solution. Slides are stained with DAPI (4′,6-diamidino-2-phenylindole) and visualized under fluorescent microscope to differentiate the fragmented (small halos/non-dispersed) form from the highly condensed chromatin (large/distinct halos). This test is performed on both neat and washed sperm, and the size of the halos is directly proportional to the DNA damage [82].

Comet Assay/Single-Cell Gel Electrophoresis (SCGE)

In this technique, DNA from the lysed sperm is subjected to agarose gel electrophoresis. The intact DNA remains inside the head of the sperm, whereas the fragmented DNA migrates and appears as a tail [83]. Fluorescent dye SYBR Green I is used for

staining, and the fragmented DNA is visualized under fluorescent microscope. The length of the tail (fragmented DNA) is an indicator of the extent of DNA damage. SCGE assay is performed on fresh semen samples, and it requires a minimum of 5000 spermatozoa. Thus, the SDF can be assessed easily in oligozoospermic samples using comet assay [84].

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL)

TUNEL assay identifies both the single- and double-strand DNA breaks in the spermatozoa from neat, washed, and cryopreserved semen samples. It is becoming a popular technique and gaining clinical importance among the other available assays used to measure SDF, for its rapid and easy procedure. DNA breaks are labeled with 2′-deoxyuridine 5′-triphosphates (dUTPs) coupled with fluorescein isothiocyanate (FITC). Incorporation of the dUTPs at 3′hydroxyl (OH) break ends of ssDNA and dsDNA is carried out by template-independent DNA polymerase known as terminal deoxynucleotidyl transferase (TdT). Further, propidium iodide (PI) is used as a counter dye to stain the nucleus. Fluorescence signals emitted are directly proportional to the DNA breaks and can be determined either by fluorescence microscope or flow cytometer [85, 86]. Detection of DNA breaks using flow cytometer is highly sensitive and most accurate technique with high reproducibility [87].

We have established TUNEL protocol for the measurement of SDF using Accuri C6 benchtop flow cytometer for clinical laboratories [88]. Initially, a reference value of 19.25% was established to differentiate healthy donors from infertile men [89]. Recently, benchtop flow cytometer was used to measure SDF in large cohort size of infertile patients ($n = 261$) and compared with proven fertile donors. The assay had a high positive predictive value (91.4%) and specificity (91.6%) with a reference value of 16.8% [90]. Apart from standardizing the threshold values for SDF, our center had also compared the SDF results for the same samples determined using Accuri C6 benchtop flow cytometer from another reference laboratory at Basel, Switzerland. The interlaboratory variation was significantly less, and both the centers had a high correlation of $r = 0.94$ [91]. Based on the reports of the several conducted experiments, a standardized, simple, and easy protocol had been proposed for SDF testing using TUNEL technique in clinical laboratories [85, 88, 89, 91].

SDF Testing for Male Infertility

Damage in the paternal genome is one of the leading causes of fertilization failure. SDF testing is an emerging and advanced tool for evaluation of male infertility. The clinical practice guideline (CPG) proposed by Agarwal et al. provides an

Table 9.2 Sperm DNA fragmentation and reproductive outcomes

Impact of high SDF on reproductive outcomes	Studies
Natural pregnancy Very low conception rates	Spanò et al. [95]
Intrauterine insemination Low pregnancy rate (OR = 9.9) Pregnancy loss with SDF > 12% and DFI > 27%	Muriel et al. [96] Duran et al. [97] Rilcheva et al. [98]
IVF/ICSI Negatively correlated with SDF Fair to poor predictive value of different SDF assays for prediction of pregnancy [100]	Cissen et al. [99]
Fertilization rate and embryo quality SDF ≥ 22.3% had significantly lower fertilization rates with ICSI Negative impact on reduced cleavage and blastulation rate decreased blastocyst development	Simon et al. [100] Morris [101] Virro and Evenson [102] Mohammad et al. [103]
Live birth rate (LBR) Negative association with LBR after IVF Increased LBR with low sperm DNA fragmentation High miscarriage rates and recurrent spontaneous abortion after IVF and ICSI	Simon et al. [104] Osman et al. [105] Robinson et al. [106]

Data from Panner Selvam and Agarwal [107]

evidence-based recommendations for the clinical utility of SDF testing in infertile men [86]. SDF testing for patients with clinical varicocele and borderline semen parameters can help the physicians for selecting these patients to restore impairments caused by varicocele and achieve better fertility outcome [86]. Additional SDF testing of ejaculated sperm in oligozoospermic patients and men with high SDF can be benefited by the use of testicular sperm for ART procedures [22, 92, 93]. Also, SDF testing is considered as a predictive tool to assess the outcomes of natural pregnancy and ART. Strengths-Weaknesses-Opportunities-Threats (SWOT) analysis revealed that CPG can be implemented in the daily routine practice for the integration of SDF testing to increase the outcome of ART [94]. Table 9.2 describes the effect of SDF on outcome of natural pregnancy and other IVF techniques.

Conclusion

In this chapter, we have provided a concise explanation of the underlying mechanisms of SDF in context to its induction via multiple factors and association of the same with male infertility. We suggest that potential diagnosis of male infertility can be achieved through assessment of SDF to bring about effective management approach to male infertility leading to satisfactory rates of successful pregnancy outcomes.

Review Criteria
Extensive literature search was performed on search engines such as PubMed, Medline, Cochrane, Google Scholar, and ScienceDirect databases. Information from the studies published for the past five decades until August 2018 were extracted. The literature search was limited only for the articles written in English language. "Sperm DNA damage and fragmentation" and "male infertility" were the main key terms used for conducting literature search. Other keywords used to retrieve relevant articles were "SDF and proteomics and metabolomics," "SDF assay," and "SDF and TUNEL." Book chapters and data published in scientific meetings relevant to sperm DNA damage were also included in this review.

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Chapter 10
The Sperm Epigenome and Potential Implications for the Developing Embryo

Emma Rae James, Timothy G. Jenkins, and Douglas T. Carrell

Key Points

- The unique nature of the sperm epigenome contributes to normal sperm function, embryogenesis, and offspring health through specific molecular patterns of DNA methylation, histone tail modifications and localization, and the presence of various RNA species.
- Epigenetic remodeling in the paternal epigenome during embryogenesis largely "erases" altered epigenetic signatures in the sperm but is not complete and may allow the sperm epigenome to directly impact the embryo and offspring.
- Sperm epigenetic signatures can be altered as a result of multiple modifiers (exposure to chemicals and toxins, lifestyle, diet, aging, etc.) during an individual's lifespan.
- Sperm DNA methylation signatures have tremendous potential in prediction and diagnosis. A recent example is the construction of a germ line age calculator capable of determining an individual's age based only on DNA methylation signatures.
- The sperm epigenome has great potential for clinical utility in both diagnosing and treating fertility.

E. R. James · T. G. Jenkins
Department of Surgery, University of Utah, Salt Lake City, UT, USA

D. T. Carrell (✉)
Departments of Surgery (Andrology) and Human Genetics, University of Utah School of Medicine, Salt Lake City, UT, USA
e-mail: douglas.carrell@hsc.utah.edu

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Introduction

Diverse cell types have specialized functions, even though all cells in an organism contain the same genomic material. For example, pancreatic cells can perform functions different from that of cardiac muscle cells, despite both containing the same template for protein expression. A major reason for the diversity in cell types and functions is the programming of specialized gene expression patterns which are largely set by epigenetic marks throughout the genome. Epigenetics is the balance of many regulatory factors that work in concert to determine expression patterns in a cell-specific manner.

"Epigenetic marks" are factors or chemical modifications that are capable of modulating gene expression through multiple mechanisms. Epigenetic marks, when taken together, form a cell-specific epigenetic signature which is maintained by proteins whose specialized function is to set and maintain these marks. By definition, epigenetic modifications are heritable, thus allowing for transgenerational inheritance of phenotypes that may originate in an individual due to specific environmental exposures or other epigenetic-modifying events. Classes of epigenetic marks include histone modifications, DNA methylation, and noncoding RNAs.

Nucleosome components, histones H2A, H2B, H3, and H4, can undergo chemical modifications, such as methylation or acetylation of lysine and serine residues on histone tails [1]. Histone tail modifications are one of the most important factors for determining the condensation of chromatin structure and subsequent accessibility of transcriptional machinery. Therefore, histone modifications help to regulate transcription by promoting either a more "open" or "condensed/closed" chromatin structure and poising genes for activation or silencing, respectively [2]. Histone modifications are mediated by specialized proteins such as histone methyltransferases, histone acetyltransferases, and histone deacetylases whose function is to add or remove histone marks as required by the cell [1].

DNA methylation is mediated by DNA methyltransferases (DNMTs). There are multiple proteins in the DNMT family, each with specialized functions for either the establishment or maintenance of DNA methylation patterns in cells [3]. DNA methylation occurs on cytosine residues at cytosine-phosphate-guanine (CpG) dinucleotides. Hypermethylation of CpG-rich gene promoters is associated with inactive genes, as DNA methyl marks restrict access of transcriptional machinery to promoters, thereby silencing gene expression [3]. In specific cases, DNA methylation patterns are inherited transgenerationally and have the ability to drive offspring phenotypes [4, 5].

Noncoding RNAs (ncRNAs) are a large class of RNA species which do not code for proteins. Subclasses of ncRNAs include micro(mi)RNAs, piwi(pi)RNAs, and tRNA-derived small RNAs (tsRNAs or tRFs), among others. These ncRNAs regulate gene expression by inducing degradation of complementary messenger(m) RNAs or by interacting with translational machinery in the cell, classifying them as epigenetic regulatory factors [6, 7]. Other epigenetic factors such as histone modifications and DNA methylation have been more extensively studied compared to

ncRNAs in many contexts; however, emerging evidence suggests that ncRNAs may play a more crucial and widespread role in cellular regulation and development than is currently understood.

Epigenetic signatures are capable of changing in response to environmental factors and disease. This prompts the question of whether these marks are heritable and if offspring could be adversely affected by epigenetic changes seen in parents. The male gamete was initially thought to be relatively void of epigenetic signatures due to it being transcriptionally inert and therefore not requiring a gene expression pattern in the same way as somatic cells. Research has shown, however, that sperm have an incredibly unique epigenetic landscape including functionally important chromatin modifications, predictable and heritable DNA methylation patterns, and a significant ncRNA payload which has only recently begun to show importance in fertility and offspring fate [8–11].

Epigenetics of the Male Gamete

Sperm chromatin is unique in comparison to chromatin contained in somatic cells. During late spermiogenesis, 90–95% of histone proteins are replaced by protamines in the male gamete. Protamines are responsible for generating the tight, toroid structure that sperm DNA forms which is necessary for both motility and protection from oxidative stress [12]. Research has shown that the 5–10% of histones retained by sperm cells occur at deliberate genomic locations in an effort to poise developmental genes for transcription in the early embryo [13]. Additionally, these retained histones undergo histone tail modifications which could provide additional regulatory functions during embryonic development [2]. Sperm DNA also contains unique methylation patterns which are relatively hypermethylated in comparison to the hypomethylated patterns seen in the female gamete. These patterns include imprinted regions which have been shown to escape the reprogramming of parental DNA methylation patterns which occurs in the preimplantation embryo [14, 15]. Paternal imprinting functionally regulates gene expression in the developing embryo [16]. Overall, there are many epigenetic factors exclusive to the male gamete which have shown importance in fertility, embryo development, and offspring health. This chapter provides a review of the current literature relating to sperm epigenetics, fertility, and embryogenesis.

Sperm Chromatin: Fertility/Embryogenesis

During the stepwise histone to protamine transition, canonical histones are first replaced by transitional proteins that are subsequently replaced by protamines (P1 and P2) (see Fig. 10.1). This replacement allows sperm DNA to be compacted up to 20 times more than DNA in somatic cells. This compaction aids in sperm motility and

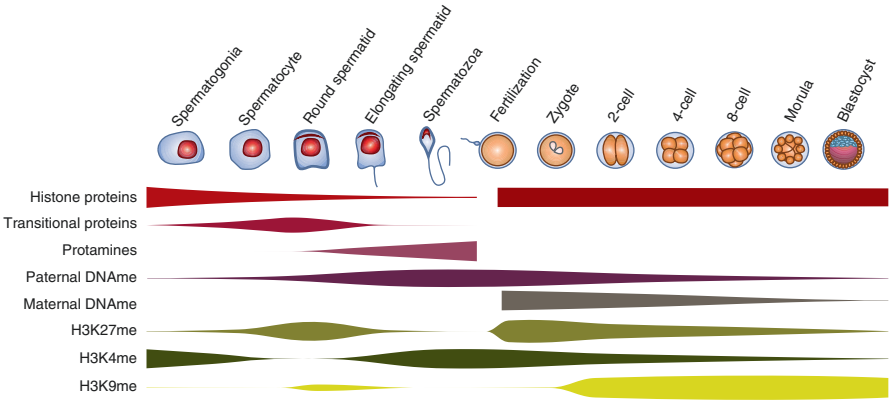

Fig. 10.1 The setting of the sperm epigenome during spermatogenesis and modifications during early embryogenesis. This figure schematically demonstrates the timelines of key remodeling events in the formation of the sperm epigenome. While the thickness of the longitudinal bands reflect the relative fluctuations in expression, they do not reflect the percentage of the genome affected or the regulation of expression of key embryonic developmental genes. As noted, large-scale remodeling events are initiated at the pronuclear stage of embryogenesis, including the active removal of most paternal DNA methylation and replacement of histones

protection from oxidative stress. P1 and P2 are found throughout the sperm genome at approximately a 1:1 ratio [12]. Normal protamination is an essential part of male fertility, and aberrations have been consistently associated with improper spermatogenesis, poor semen parameters, reduced fertilization capabilities, and reduced implantation rates [17–19]. It has been demonstrated that both altered P1/P2 and histone/protamine ratios are associated with reduced male fertility. In patients undergoing IVF treatment, altered P1/P2 ratios were strongly correlated with increased sperm DNA damage as well as impaired fertilization capabilities [20]. In a mouse model, intracytoplasmic sperm injection (ICSI) has been utilized to ensure successful fertilization using sperm containing altered histone/protamine ratios and high DNA fragmentation. Interestingly, these zygotes failed to develop into viable embryos [21]. Similar results from a number of studies show a strong correlation between sperm nuclear landscape and fertility [12, 20–23]. Likewise, sperm populations containing aberrant protamination commonly show high correlations with DNA fragmentation. These correlations suggest that abnormal sperm chromatin structures are more susceptible to DNA damage, likely due to improper protamination. Together, this evidence conveys that protamination is an essential consideration when evaluating male factor infertility.

Outside of high DNA fragmentation levels, an additional explanation for the requirement of proper protamination has been proposed. While 90–95% of histones are replaced by protamines in the sperm cell, 5–10% still remain, prompting the question of whether these histones have functional consequences during fertilization and embryogenesis. Recent work has demonstrated that retained histones are found at consistent and deliberate locations throughout the sperm genome. Histones are seen at developmental genomic locations, poising these genes for activation dur-

ing early embryogenesis [13]. These findings imply that proper protamination, and likewise normal retention of histones, is not only important in regards to male factor infertility as a reflection of abnormal spermatogenesis but also in terms of paternal contributions to embryogenesis.

Histone tail modifications are a major class of epigenetic regulators in somatic cells. While sperm contain a very low abundance of histone proteins as compared to somatic cells, retained histones in sperm still undergo modifications which may have important regulatory functions. In somatic cells, acetylation of H3 and H4 as well as methylation of H3K4 helps to drive a chromatin state which poises genes for activation. Conversely, methylation of H3K9 and H3K27 and deacetylation of H3 and H4 drive a chromatin state which silences genes at those locations [1, 24]. Histone modifications are capable of regulating transcription individually; however, most often a combination of modifications works in concert to drive the transcriptional state necessary for either gene activation or silencing. In sperm, perturbations to histone methylation and acetylation result in varying degrees of fertility loss, including impaired spermatogenesis and sterility [25]. While the mechanism accounting for these observations is unknown, the ability of histone modifications to drive chromatin state changes that can regulate transcription suggests that poising of genes by epigenetic marks in sperm may contribute to fertility and development [24]. In support of this, infertile men have been shown to display altered genomic distribution of histones, as well as alterations in H3K4 and H3K27 methylation [26].

Recent evidence from zebra fish studies suggests that sperm chromatin composition and histone modifications may also be capable of regulating transcription in the early embryo via more indirect methods. Histone H2A variant H2A.Z(FV) exists in both human and zebra fish sperm [13, 27]. A recent study found that genomic regions of zebra fish sperm containing both H2A.Z(FV) and H3K4 methylation almost completely lack DNA methylation. Perturbations affecting H2A.Z(FV) placement resulted in the accumulation of DNA methylation at previously hypomethylated regions [28]. This study proposes that H2A.Z(FV) acts as a "placeholder" nucleosome which in combination with H3K4 methylation prevents DNA methylation, thereby poising genes for activation during embryogenesis. This hypothesis is further supported by the regions in which H2A.Z(FV) is found. The "placeholder" nucleosome occupies regions encoding for housekeeping genes and early embryonic transcription factors [28]. While chromatin composition varies greatly between species and therefore this mechanism may not be conserved in the human germline-to-embryo transition, it provides additional evidence for the importance of sperm nuclear landscape and epigenetic marks in development.

DNA Methylation: Fertility/Embryogenesis/Inheritance

DNA methylation is an additional layer of epigenetic regulation seen in the male gamete that shows significant promise in affecting fertility and embryogenesis. Many associations between DNA methylation alterations and various phenotypes,

including reduced fertility, have been explored. Early studies have observed DNA methylation aberrations in sperm with abnormal chromatin packaging, sperm from men who generate poor quality in vitro fertilization embryos, as well as infertile men [26, 29–32].

In addition to associations between aberrant DNA methylation and infertile groups, this epigenetic mark also carries the ability to regulate the developing embryo. While there are many observations of this, an interesting example relates to genomic imprinting. Prior to zygotic genome activation in the early embryo, DNA methylation patterns acquired from parents are either active or passively removed [14]. This process led to the belief that DNA methylation of sperm was not an epigenetic mechanism capable of inducing heritable changes. However, imprinted regions of the sperm genome manage to escape this reprogramming event in early embryos, and these regions maintain the methylation signature provided to the embryo by sperm [14, 15]. This provides evidence that sperm DNA methylation could, in fact, drive epigenetic inheritance in offspring.

Additionally, paternally imprinted regions are associated with genes necessary for development, and therefore imprinting allows these genes to be poised for activation or repression in the early embryo [16]. This evidence suggests that methylation signatures in sperm at paternally imprinted regions are important for normal embryogenesis. In support of this, one study completed DNA methylation analysis on sperm from men who experienced idiopathic, recurrent pregnancy losses with their partners. This study found a loss of imprinting, characterized by reduced DNA methylation levels at the H19 imprinting control region (ICR). H19 is only expressed by the maternal allele; therefore, in sperm, the H19 ICR is normally hypermethylated which leads to silencing of this gene on the paternal allele [16]. These results suggest that sperm DNA methylation and genomic imprinting are important in modulating the expression of genes in a parent-of-origin manner and that a loss of paternal imprinting is associated with recurrent pregnancy loss in couples.

Associations between aberrant sperm methylation patterns and embryogenesis have been shown in contexts other than recurrent pregnancy loss as well. A recent study set out to determine whether sperm methylation patterns could be predictive of male fertility and embryo quality during in vitro fertilization (IVF) treatment. IVF patient groups were determined based on whether their sperm generated good quality embryos and positive pregnancies or generated poor-quality embryos and negative pregnancies. These two groups were compared to known fertile men. This study found that predictive models based on methylation array data from these groups were highly predictive of male fertility status. Additionally, hierarchical clustering was capable of identifying clusters containing IVF patients and poor embryo quality samples based on methylation array data. While the methylation changes observed between these groups were not biased toward genomic regions of any particular annotation category – such as imprinted regions – these data show that global alterations in sperm methylation can be predictive of male fertility status and potentially embryo quality during IVF treatment [31].

An additional mechanism by which DNA methylation can affect the embryo and offspring is by the transmission of heritable phenotypes via an epigenetic mecha-

nism. Genomic imprinting is evidence that the reprogramming of methylation signatures during early embryogenesis is not complete. Therefore, it is possible that methylation signatures beyond imprinted regions may be provided to the developing embryo by sperm with the purpose of modulating development and subsequent phenotype both inter- and transgenerationally. In fact, some of these regions have been identified [33–35]. The most well-documented example of transgenerational inheritance is seen in agouti mice. Agouti mice are characterized by a yellow coat color, diabetes, and high susceptibility to tumors. Regulation of this phenotype occurs via DNA methylation-induced modulation of intracisternal A particle (IAP) elements in the context of *A* or *Axin1* genes in the mouse genome. Induced DNA methylation alterations at this region can be seen through multiple generations, providing proof that transgenerational epigenetic inheritance via DNA methylation does, in fact, occur [4, 5].

The agouti mouse model of transgenerational epigenetic inheritance is not specific to spermatozoal DNA methylation patterns. Therefore, the question remains as to whether paternal DNA methylation patterns are capable of transmitting a phenotype to offspring. Implications of paternal diet have been studied in various models, including rats. One such study discovered that the female offspring of male rats consuming a high-fat diet displayed multiple characteristics consistent with metabolic phenotypes. Additionally, these observations were noted in both F1 females and female offspring generated from F1 males (F2 females) [36]. This evidence suggests a transgenerational transmission of the metabolic phenotype. These female offspring displayed reduced birth weight, decreased pancreatic beta-cell mass, and glucose intolerance. DNA methylation analysis was conducted on the sperm from the F0 high-fat diet-fed rats as well as the F1 males, and multiple alterations were observed when compared to control rats. Numerous of the same differentially methylated regions were observed in both the F0 and F1 male sperm, suggesting a possible mechanism for transgenerational inheritance of metabolic disease. This group also observed differential expression of spermatozoal RNAs in rats consuming a high-fat diet, proposing a potential additional or supplementary mechanism for the inheritance shown [36].

Taken together, these studies – as well as many others – provide evidence that DNA methylation in the male gamete is an important consideration in many regards. Male fertility, embryo health, and quality and offspring phenotype are all areas which may be heavily affected by the DNA methylation patterns seen in sperm.

ncRNAs: Fertility/Embryogenesis/Inheritance

Along with mounting evidence that sperm DNA methylation plays a functional role in embryos, revolutionary studies have determined that sperm contain multiple RNA species that are stable in the embryo following fertilization [37, 38]. These include mRNAs that are left over from spermatogenesis and have provided insight into events occurring during this process [37, 39]. Sperm also contain mRNAs

which may be functionally important to the developing embryo and for male fertility [39–41]. In addition to mRNAs, sperm contain comparatively high levels of noncoding RNAs [9]. Recent studies have provided new and exciting evidence that the noncoding RNAs contained in sperm may contribute to regulation in the developing embryo [8, 10]. Additionally, spermatozoal RNAs have been implicated in a newly hypothesized model of epigenetic inheritance of metabolic disease [42–44].

A recent study tracked and described the biogenesis of spermatozoal RNAs. The RNA payload of sperm cells is established in two major waves in the mouse. The first occurs in the testis and results in an RNA payload comprised mostly of piRNAs left over from spermatogenesis. The second wave occurs during epididymal transit and results in dramatic reprogramming of the sperm RNA signature. At the end of epididymal transit, mature sperm cells contain various noncoding RNA species, with a bias toward tRFs and miRNAs. Reprogramming of the sperm RNA signature during epididymal transit is mediated by epididymosomes. Epididymosomes are exosomes which are released by the epididymal epithelium and deliver cargo to developing sperm cells. Among this cargo, which is primarily proteins necessary for sperm maturation and motility, is an RNA repertoire which closely mirrors that of mature sperm cells. Surprisingly, mature sperm isolated from the cauda epididymis and sperm isolated from the testis share many species of tRFs and miRNAs, despite the reprogramming that occurs during epididymal transit. Sperm isolated from the caput epididymis, however, lack many of the RNA species shared between testicular and cauda sperm [11]. These findings have prompted the hypothesis that sperm undergo either a random or programmed loss of RNA species following development in the testis and subsequently reacquire the RNA species seen in mature cells via epididymosomes during epididymal transit.

The RNA species that are lost and subsequently regained by sperm cells have been implicated in improper embryonic implantation as well as gross defects in embryonic development. A recent study used testicular, caput, and cauda sperm to generate mouse embryos. Surprisingly, caput-derived embryos showed significantly reduced rates of successful implantation and gross morphological defects and ultimately did not develop into viable offspring [8]. The group conducting this study hypothesized that these changes were due to caput sperm's RNA signature, which lacks many miRNA and tRF species found in both testicular and cauda sperm. To test this, the group isolated total sRNA, miRNA, and tRF fractions from cauda epididymosomes – which would normally be delivered to caput sperm during epididymal transit – and microinjected these fractions, separately, into caput-derived zygotes. They observed a "rescuing" of gene expression profiles in the caput-derived embryos injected with the miRNA fraction. No change was seen in gene expression of the caput-derived zygotes injected with the tRF fraction. These results suggest that the miRNAs delivered to sperm during epididymal transit are required for proper preimplantation gene expression in the mouse [8]. Additionally, the total sRNA-injected zygotes were cultured to blastocyst stage and then transferred to pseudopregnant surrogates. Once again, a rescuing event was observed, and the microinjected caput-derived embryos successfully developed and did not suffer the embryonic lethality observed in caput-derived embryos [8]. Of the

miRNAs implicated by this group, miR-34c has previously been shown by another group to be essential for the first cleavage division in mouse embryos. In that study, miR-34c was observed in mature sperm and zygotes, but not in oocytes or preimplantation embryos, providing evidence that miR-34c expressed in zygotes is delivered to the oocyte by sperm. Following microinjection of a miR-34c inhibitor into zygotes, this group observed that over 70% of zygotes failed to cleave, as compared to 97% cleavage in controls. This evidence suggests that sperm-borne miR-34c is required for the first cleavage division in mouse [10]. Taken together, these two studies strongly suggest a role for spermatozoal RNAs, specifically miRNAs, in embryogenesis.

Similar to a previously mentioned study on DNA methylation, multiple studies have also proposed a role for spermatozoal RNAs in epigenetic inheritance of metabolic disease from fathers. Altered metabolic phenotypes have been observed in the offspring of male mice consuming high-fat or low-protein diets. These offspring display a phenotype characterized by glucose intolerance and impaired insulin secretion [36, 42, 43, 45, 46]. In an effort to understand the mechanism by which this transmission occurs, multiple groups have focused their attention on spermatozoal RNAs as potential regulators of this inheritance. The sperm of male mice and rats consuming either a high-fat or low-protein diets display unique RNA signatures as compared to controls, including changes in abundance of some RNA species as well as increased levels of modified tRFs [42–44]. The tRF modifications observed at increased levels in altered-diet sperm are mediated by DNA methyltransferase 2 (DNMT2). Following DNMT2 knockout in male mice, these tRF modifications are no longer observed on tRFs contained in sperm. Interestingly, the offspring of DNMT2$^{-/-}$, altered-diet males do not display the metabolic phenotype seen in the offspring of DNMT2$^{+/+}$, altered-diet males. This evidence suggests that DNMT2-mediated tRF modifications in sperm may contribute to epigenetic inheritance of metabolic disorders [42, 44]. A study conducted by a different group worked to determine whether changes in abundance of some RNA species seen in sperm of altered-diet mice could be responsible for changes in gene expression observed in altered-diet-derived embryos. This group generated altered-diet-derived zygotes which were subsequently microinjected with RNAs isolated from control-diet sperm. The resultant embryos displayed gene expression patterns which mimicked that of controls [43]. These results suggest that spermatozoal RNA content is capable of shaping expression patterns in the embryo and propose that abnormal abundance of RNAs in sperm could be a driver of epigenetic inheritance.

Clinical Relevance

While many questions still remain, it is clear from the currently available data that epigenetic patterns in sperm offer some level of clinical utility. This may be valuable from the diagnostic perspective and potentially even in the development of treatments for male infertility.

One area of significant optimism is in using deep learning approaches to predict reproductive outcomes based on sperm epigenetic signatures. Recent studies have shown that sperm epigenetic signatures are quite capable of this sort of prediction, most notably with an analysis of aging. Specifically, this study described the construction of a model utilizing sperm DNA methylation patterns that can predict an individual's age with a high degree of accuracy [47]. While not yet proven, it is reasonable to assume that the signatures of aging in sperm are correlated to offspring phenotypic outcomes, which have been identified in previous studies work. Specifically, it has been shown that there is an increased incidence of neuropsychiatric abnormalities in the offspring of older fathers. Thus, the ability to predict age and age acceleration using epigenetic signatures in sperm may offer valuable information about risks to the offspring (and thus diagnostic utility), though more work is needed to establish this. Similar deep learning approaches can be taken to predict the likelihood of success in any given treatment course (IUI, IVF, etc.), and patients can use these data to help inform family planning decisions, and clinicians could use these data to help guide clinical decision-making. Some work has already been performed in this regard with both DNA methylation and RNA [31, 40]. Both of these studies suggest that such epigenetic diagnostic approaches are realistic and are not far off.

The potential therapeutic utility of the sperm epigenome is clear, though, for the most part, the required technology is not yet fully developed despite rapid advancements. Genome editing does offer some hope to correct highly abnormal DNA methylation signatures in gametes should they be identified, but the technology requires further development to make this reasonable for DNA methylation correction and for more widespread and regional application (not just at a single locus). There are some potential interventions identified by recent work that would be well suited for a more rapid application in the market. Most notably among these is the potential utility of microRNA incubation prior to IVF or ICSI. This theoretical approach is based on the data which suggest that RNAs are added to the sperm in the epididymis and that the addition of these RNAs impacts fertilization, embryo development, and even offspring health. If alterations to the abundance of these RNAs can be reliably identified, it is not unreasonable to assume that these could be supplemented in an incubation step prior to IVF or ICSI to improve embryogenesis and offspring health outcomes.

More work is required to enable the utilization of sperm epigenetic marks in the clinic with some aspects of diagnostics and therapeutics being closer to fruition than others. Despite the great deal of effort that is still needed, the potential clinical utility of the sperm epigenome in reproductive medicine is clear and warrants the required input.

Review Criteria

A thorough search of the literature was performed using PubMed and Google Scholar. We used the search terms "sperm epigenetic," "sperm DNA methylation," "sperm RNA," "sperm histone," "sperm chromatin," "protamine," "transgenerational epigenetics," "embryo epigenetics," and "male infertility epigenetics" for study identification and data extraction.

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Part III
Clinical Case Scenarios

Chapter 11
Klinefelter Syndrome

Marlon P. Martinez, Haitham Elbardisi, Ahmad Majzoub, and Mohamed Arafa

Key Points

- The variants of KS share the same features of hypergonadotropic hypogonadism but present with more multifaceted physical, medical and psychological features.
- Learning and behavioural difficulties can be seen at an earlier age, while androgen deficiency and infertility are usually encountered during adulthood.
- TRT addresses hypogonadism during puberty to enhance the quality of life and prevent the long-term complications of androgen-deficient states.
- Micro TESE provides significantly superior overall outcome compared to other sperm retrieval techniques.
- Men with sex chromosomal abnormalities have low or no increased risk of producing offspring with the same abnormalities after ICSI.

Case Scenarios

Case 1

A 16-year-old boy was brought to a paediatric clinic due to delayed pubertal development. His parents noted that he did not grow up the same with his peers and that there was sparse hair on the face, body and axilla, which was noted to appear when

M. P. Martinez
Philippine Urological Association, Department of Surgery, Section of Urology, University of Santo Tomas Hospital, Sampaloc, Manila, Philippines

H. Elbardisi · A. Majzoub · M. Arafa (✉)
Department of Urology, Hamad Medical Corporation, Doha, Qatar

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he was 14 years old. He was noticed to have learning and behavioural struggles. At one time, speech therapy was required to cope with his age. He had difficulties at school when he was 8 years old. He was always enrolled in remedial classes. Physical examination revealed the following anthropometric measurements: height = 190 cm (>95th percentile), weight = 85 kg (>95th percentile) and arm span = 201 cm. There was bilateral gynaecomastia. Both testes measured 2.5 cm on the long axis. There was minimal coarse pigmented pubic hair at the base of the penis. Serum total testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were all normal. Chromosomal examination showed 47,XXY.

Case 2

A 34-year-old businessman presented to the clinic to establish primary care for infertility. He was happily married but had been unable to conceive over the last 5 years. His wife was 27 years old and had regular check-ups with her fertility doctor. He claimed that he had a feeling of daily extreme fatigability. Despite this, he reported having normal sexual function. On physical examination, both testicles were firm and measured 2.0 × 1.5 × 1.0 cm. The rest of the findings were unremarkable. Hormone testing revealed the following results: FSH = 34.7 mU/ml (N 1.5–12.4 mU/ml), LH = 22.3 mU/ml (N 1.8–10.8 mU/ml) and total testosterone = 156 ng/ml (N 193–824 ng/ml). Two semen analyses both showed azoospermia. A karyotype study was requested that showed 47,XXY.

Introduction

Sexual chromosomal aneuploidies, including tetrasomy and pentasomy anomalies, may be observed in 1:18000–1:100000 male births [1]. Among these abnormalities, Klinefelter syndrome (KS) with a karyotype showing 47,XXY appears to be the most common, where an error of nondisjunction during gametogenesis provides the extra X chromosome [2].

Historical Background

As early as 1842, Lereboullet presented the case of two brothers with bilateral gynaecomastia and small testes [3]. KS was not studied extensively at that time until in 1942, when a travelling medical fellow from Massachusetts General Hospital named Dr. Harry Klinefelter gave meticulous attention to patients who had a similar clinical presentation. He initially saw a tall black boy who had gynaecomastia and a 1.0–1.5-cm-long testis. Additional eight patients with varied ages of 17–38 years

consulted for the same condition. A high level of FSH in urine was excreted excessively in all patients. Azoospermia was observed in all patients with varying degrees of advanced hyalinization of tubular elements observed in testicular biopsies. Dr. Klinefelter was fortunate enough to be allowed by his consultant, Dr. Fuller Albright, to use his surname to name this collection of signs and symptoms; hence, the term Klinefelter syndrome rose [4].

In 1956, Bradbury et al. identified female chromatin in an oral smear from a 19-year-old male presenting with the same clinical condition [5]. Testicular biopsies revealed tubular sclerosis with normal Leydig cells, while oral smears showed the genetics of a female.

It was not until 1959 that Jacobs and Strong found the chromosomal components of KS [6]. They presented a case of a 24-year-old male with a high-pitched voice, poor facial hair growth, small testes and gynaecomastia. Samples from a sternal marrow puncture were used for the investigation of somatic chromosomes. They found that this patient with gonadal dysgenesis had an additional chromosome within the same size range as the X. As a result, the genetic constitution of XXY was linked to KS.

Incidence of Klinefelter Syndrome

Patients with KS are diagnosed prenatally in only 12% of cases, while 25% of cases are detected during childhood and adolescence. Unfortunately, approximately 65% of KS patients will be undiagnosed in their lifetime [7]. In a national registry study in Denmark, a prenatal examination of male foetuses resulted in a prevalence of 153 per 100,000 males [8]. By contrast, approximately 40 per 100,000 males were diagnosed postnatally, of which only less than 10% were diagnosed before puberty.

Newborn screening for methylated FMR1 DNA performed in America revealed an incidence rate of KS of 1 in 633 newborns [9]. Out of a sample of 16,252 white men, 27 were identified with KS, while 20 African American men showed positive results for KS out of a sample of 10,979. Three from a sample of 5396 Hispanic men and another 3 from a sample of 847 Asian men likewise revealed positive results for KS.

KS is the most common chromosomal aneuploidy in infertile men with testicular failure, with a prevalence of 10% in azoospermic men and 0.7% in oligozoospermic men [10–12]. A recent study from the Middle East [13] reported an incidence of 3.7% for KS among patients with severe oligozoospermia and azoospermia, implying a possible effect of ethnicity on the prevalence of KS.

The classic form (47,XXY) of KS makes up 80–90% of the cases, while other forms of aneuploidies constitute the remaining 10–20%, including mosaicisms, higher-grade aneuploidies and abnormal X chromosome structure [14]. Of the higher form of KS variants, 48,XXYY occurs in 1:18,000–1:40,000 male births [15], 49,XXXXY occurs in 1:85,000–1:100,000 and 48,XXXY occurs in 1:50,000 [16].

Genetic Basis of Klinefelter Syndrome

Mechanisms Causing Klinefelter Syndrome

The 47,XXY condition has been extensively explored. Previous molecular studies revealed that the majority of human trisomies were derived primarily from maternal meiosis errors [17]. In the case of KS, the trisomy is paternally derived in approximately 53.2% of the cases (Fig. 11.1). Trisomies can only arise by an error in meiosis I, unlike in maternal origin (Fig. 11.2), wherein these can evolve from errors in meiosis I (34.4%) and meiosis 2 (9.3%) or postzygotic mitotic errors (3.4%) [18].

During gametogenesis in both males and females, highly specialized cell division processes occur, consisting of one cycle of DNA replication followed by another two stages of cell division comprising meiosis I and 2, resulting in the generation of haploid gametes. The pairs of homologous chromosomes synapse during the prophase of meiosis I. As a result, chiasmata are formed at the exchange sites. These chiasmata display very important functions in the proper disjunction of chromosomes during division in meiosis I [19]. An error of nondisjunction during paternal gametogenesis provides the extra X chromosome in KS patients [2].

Several theories were discovered to explain the impact of paternal or maternal origin of the supernumerary X chromosome in KS on the phenotypic features of KS. The inactivation of one of the X chromosomes of normal females during transcription will compensate for the minimal gene content of the male Y chromosome. Among these genes that undergo inactivation is the androgen receptor gene. The length of a stretch containing polyglutamine coding repeats, (CAG)n, is valuable because its length is inversely proportional to its activity [20].

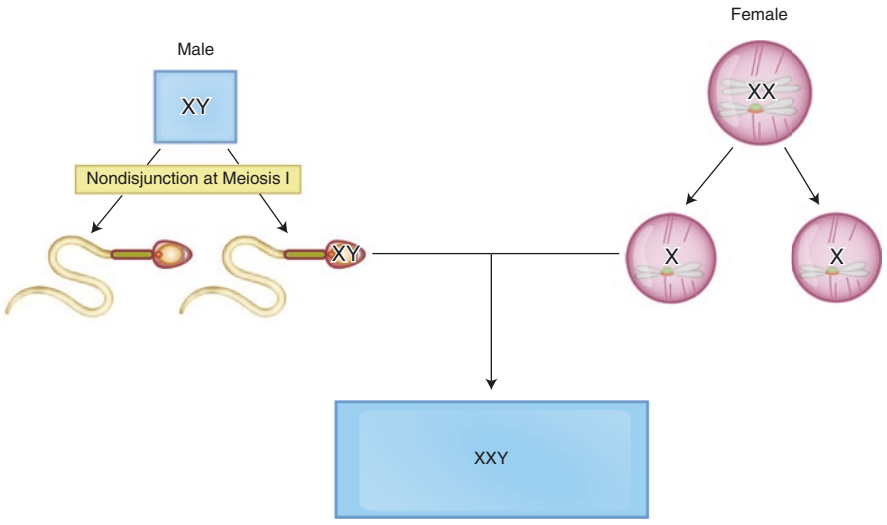

Fig. 11.1 Paternal origin of 47,XXY. Nondisjunction at meiosis I

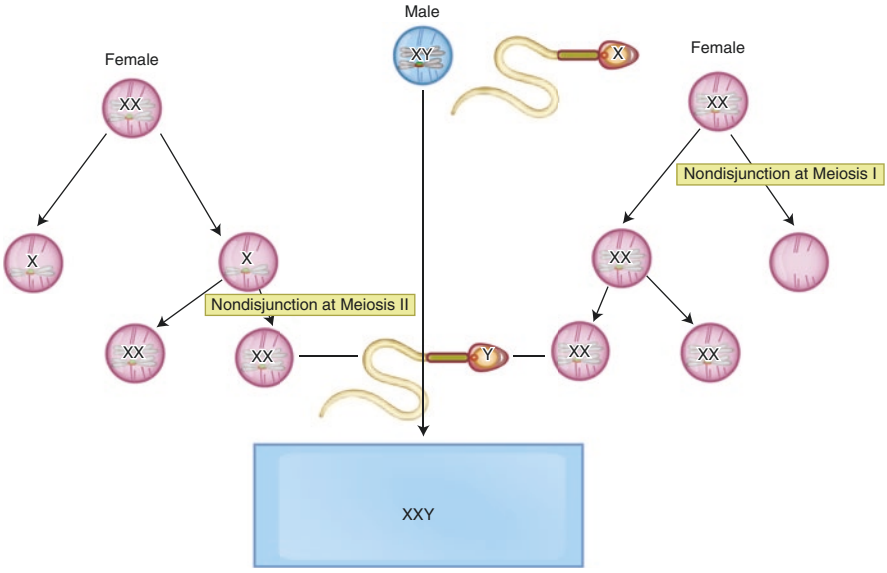

Fig. 11.2 Maternal origin of 47,XXY. Nondisjunction at meiosis I and II

Table 11.1 Variants of KS

Karyotype variants of Klinefelter syndrome
46,XX/47,XXY
48,XXYY
48,XXXY
49,XXXXY
47,XY,i(X)(q10)

Maternal age is one of the proven risk factors for KS, where the prevalence of KS increases four times with a maternal age above 40 years. By contrast, studies failed to find such correlation with paternal age [8, 21].

Karyotype Variants of Klinefelter Syndrome

Many different karyotypes of KS have been reported in the literature (Table 11.1). 46,XX/47,XXY is the most frequent variant of KS. Although patients with this variant tend to have larger testes due to the presence of spermatogonia with normal chromosomal constitution, the absence of sperm in the ejaculate is present in half of the cases. Men with 46,XX KS share similar clinical symptoms with those with 47,XXY but often present with normal height and intelligence [22].

Other variants of KS have the same hormonal prolife of elevated gonadotrophins and decreased testosterone levels. However, more multifaceted physical, medical and psychological features make this variant unique from the more common 47,XXY

and increase the risk for congenital malformations [1]. These variants include 48,XXYY, 48,XXXY and 49,XXXXY. 48,XXXY is the most common variant, with an incidence of 1:18,000–1:40,000 male births [15]. Testicular dysfunctions are more noticeable during adolescence because androgen deficiency sets in and gonadal fibrosis begins to occur. Microorchidism appears in all of these variants [23].

Different variants of KS have their own unique physical and behavioural profiles. Among these high-order variants, men with 49,XXXXY have the shortest stature, possibly due to tremendous overdosage of autosome genes disturbing the growth pathway and the development of many organ sites [24]. In addition, a more distinctive degree of facial dysmorphism and a more common congenital malformation are noted in this variant. 48,XXYY patients tend to have more pronounced abnormalities than 47,XXY KS patients, including clinodactyly, radioulnar synostosis and craniofacial dysmorphism. They are also more prone to neurological and upper respiratory diseases. 48,XXXY patients may show disfigured facial features, such as simplified ears, hypertelorism and prognathism. They possess other anomalies, including clinodactyly and abnormalities of the elbows, in addition to abnormal glucose tolerance.

Visootsak et al. examined the behavioural similarities and differences in individuals with 48,XXY compared to individuals with 48,XXXY and 49,XXXXY [25]. They found that men with 48,XXYY have superior overall adaptive scales in daily living skills, socialization and communication compared to their counterparts. By contrast, men with 48,XXXY and 49,XXXXY had behaviour that was inappropriate for their chronological age due to lower cognitive level.

A very rare variant of KS, 47,XY,i(X)(q10), has been reported in a 32-year-old male complaining of infertility [26]. Patients with this variant are noted to have an additional isochromosome composed of the long arm of the X chromosome. This Xq isochromosome is believed to be derived from the non-division of the centromere or sister chromatid exchange of one X chromosome [27]. They are observed to have shorter stature while acquiring normal intelligence. No successful sperm retrieval was ever reported up to this time. Due to very limited cases ever reported with this variant, some of its features are still unclear.

Effects of Klinefelter Syndrome on Spermatogenesis and Hormones

Infertility is one of the hallmarks of KS, as reported from its first description in 1942 [4]. Almost all patients were stated to be azoospermic. However, there are reports indicating that spermatozoa may be detected in the ejaculate of KS men [28].

Several hypotheses appear to explain the defective spermatogenesis in KS patients. A study by Sciurano et al. concluded that the most likely origin of spermatogenetic foci came from the clones of spermatogonia that were randomly lost on one of the X chromosomes [29]. It is believed that this happened when increased mitotic activities occurred. This implied the strongest evidence of the testicular

environment hypothesis. Another explanation is the potential complete meiosis of KS spermatogonia despite having a degree of Sertoli cell secretory dysfunction [30].

KS men might present with a progressive derangement of spermatogenesis. No large longitudinal study was reported on the diminution of spermatogenesis throughout adulthood. It is believed that spermatogenesis is relatively intact during infancy and early adolescence with progressive hyalinization occurring with age, resulting in the classic picture of KS in adult males. In one case report [31], a 34-year-old man with non-mosaic KS presented with azoospermia after 2 years of oligoasthenozoospermia and was able to produce a child. In contrast, Gies et al. reported seven non-mosaic KS teenagers (>10 years) who underwent testicular tissue recovery [32]. No spermatogenesis was found by testicular biopsy, electroejaculation or spermaturia. Therefore, these authors are not recommending cryopreservation in this age group.

Selice et al. studied semen samples from 84 non-mosaic KS men [33]. Spermatozoa in the ejaculate were found in 8.3% of patients, although a sperm concentration less than 1 M/ml was noted in this set of patients. Nine out of 24 men who underwent microdissection testicular sperm extraction (TESE) had successful sperm retrieval. In another study to determine the presence of spermatogenesis in men with KS, nine patients with non-mosaic KS and NOA underwent diagnostic TESE [34]. Cytological analysis revealed very low ratios of spermatozoa/pachytene spermatocytes in two patients. Neither the maternal nor paternal origin of the extra X chromosome could predict the presence of spermatogenesis.

In contrast, the hormonal function of KS men seems to be similar to that of the healthy population until puberty [35]. It was noted that their hypothalamic–pituitary gonadal axis functions normally during childhood and early puberty, allowing the secondary sex characteristics to develop. However, towards late puberty, FSH and LH start to rise above normal levels with a concomitant decrease in testosterone. Others may present with different hormonal profiles and phenotypic features.

As for other hormones, inhibin B seems to be within normal levels in prepubertal boys and increases steeply before the onset of clinical puberty but decreases gradually during late puberty and adulthood [36]. Serum oestrogen levels are elevated in boys with KS, especially before 12 years of age, compared to their healthy counterparts ($p < 0.0001$) [37]. The ratio of oestrogen to testosterone was increased but did not show a significant difference.

Clinical Presentation of Klinefelter Syndrome

There is wide variation in clinical presentation between adolescent and adult men with KS. At an earlier age, learning and behavioural challenges are usually encountered. By contrast, androgen deficiency and infertility are common presentations during advanced age.

Pacenza et al. reported the different clinical presentations of 94 KS patients according to age [38]. The most prevalent age at diagnosis was 11–20 years. 47,XXY was detected in 83.7% of the patients, while 47,XXY/46,XY was detected

in 7.1% of the patients. The most prevalent findings in prepubertal aged patients were cryptorchidism (55.5%), neurodevelopmental disorders including behavioural and learning difficulties (44.4%) and small testes (16.7%). By contrast, the most common presentations in pubertal patients were small testes (76.9%), neurodevelopmental disorders (53.8%) and gynaecomastia (42.3%). In all prepubertal boys, FSH, LH, testosterone, inhibin B and AMH were normal. FSH ($p < 0.001$) and LH ($p < 0.001$) showed negative correlations with age. In adult patients with KS, the most prevalent complaints were infertility (34.8%) and small testes (34.8%). Genital evaluation revealed small testes in all adult patients, varicoceles (23.3%) and gynaecomastia (31.3%). FSH was elevated in all adult patients, while LH was elevated in 83%. A negative correlation was noted between total testosterone and age ($p < 0.001$). Azoospermia was found in 89.3% of patients.

In a self-administered Web-based survey containing quantitative measures and open-ended questions regarding the present impact on the life of a total of 310 adolescents and adults with non-mosaic KS, 31% specified that infertility was their utmost challenge, while 27% cited psychological traits [39].

Other clinical manifestations of KS include the characteristic tall stature with increased span of height due to the delayed closure of the epiphyses secondary to hypogonadism. This is coupled with a decreased mineralization of bones and osteoporosis, putting KS patients at an increased risk of osteoporotic fractures. Additionally, there is a decrease in lean body mass with an increase in body fat deposition secondary to hypogonadism that may occur through a direct influence of the chromosomal defect, as these changes can be found before puberty.

KS patients are at an increased risk of multiple comorbidities. Metabolic syndrome was found in 44% of KS patients in comparison to 10% in the normal population [40]. Similarly, diabetes mellitus was reported in up to 50% of KS patients [41], together with an increase in low-density lipoprotein and a decrease in high-density lipoprotein [42], thus increasing the risk of ischaemic heart disease. This risk is even higher with the reported clotting defects in KS patients due to dysfunctional fibrinolysis secondary to the increased activity of plasminogen activator inhibitor-1, increasing the incidence of embolic events in these patients [43]. Another cardiovascular comorbidity that is prevalent in KS patients is mitral valve prolapse, with an incidence of 55% compared to 6% found in the normal population [44]. KS patients are also at an increased risk of developing different cancers, namely, breast cancer, non-Hodgkin lymphomas and lung cancers [45].

Management

Management of Hypogonadism

Hypergonadotropic hypogonadism is the hormonal outcome of KS [4]. Hypogonadism does not usually manifest until puberty sets in. However, the onset of androgen deficiency is variable among KS men. Testosterone replacement ther-

apy (TRT) is valuable for the improvement in the symptoms of androgen deficiency. It is generally believed that TRT will enhance the quality of life and prevent the long-term complications of hypogonadism. It is therefore recommended that KS men be offered TRT [46]. Currently, no specific TRT protocol for KS is available. In addition, there are no available large randomized placebo controlled trials on this matter.

There are various pharmaceutical forms of TRT, including oral, transdermal and intramuscular administration. Different preparations were used in different studies reporting its effectiveness in KS men with hypogonadism.

In an earlier study, oral testosterone was used to treat androgen-deficient KS men. In a study by Nielsen et al., 30 KS men were treated with testosterone enanthate 110 mg, testosterone propionate 25 mg or testosterone undecanoate 40 mg with an average follow-up of 3.6 years after treatment [47]. Overall, testosterone treatment had positive effects in 77% of men, while no effect was noted in 17% of men taking the medication. Beneficial consequences included an improvement in strength, sleep, sexual drive, concentration, learning ability and mood.

Other forms of testosterone have been used to treat KS. Meikle et al. reported the use of transdermal testosterone nightly for 6 months for the treatment of 13 KS men [48]. A normal range of morning serum testosterone was attained in all patients after treatment. Another study on the daily use of testosterone gel (1%) in the treatment of 86 adolescents (12–17 years) with KS was reported by Rogol et al. [49]. Testosterone and dihydrotestosterone levels increased 1.8- to 2.3-fold, while oestradiol levels increased 1.4-fold after 6 months of treatment.

A retrospective cohort analysis on the safety and efficacy of TRT in 110 adolescents with KS (10–21 years) was reported by Mehta et al. [50]. TRT included topical testosterone ($n = 104$), injectable testosterone ($n = 5$), subdermal pellets ($n = 1$) and aromatase inhibitor ($n = 75$). An improvement in average serum testosterone (from 240 ng/ml to 650 ng/ml) was noted after treatment. There was no associated suppression of endogenous serum FSH and LH. Adverse events were limited to the appearance of acne.

TRT is considered the mainstay treatment for androgen-deficient KS men. This treatment is relatively safe with minimal serious adverse events reported. However, good methodological studies are warranted to determine the optimal treatment and follow-up of these patients.

Management of Fertility

Genetic Counselling

Genetic counselling for KS patients is essential in many situations, including prenatal, paediatric adolescent and adult diagnosis. Groth et al. recommended that the care of KS men should be provided by a multidisciplinary team composed of paediatricians, speech therapists, general practitioners, psychologists, infertility special-

ists, urologists and endocrinologists [51]. Provided care should focus not only on infertility issues but also on the impact on the quality of life and overall health status.

Currently, infertile men have the chance to have biological children due to widespread application of assisted reproductive technology (ART). Most KS men are azoospermic [4]; therefore, their chance to have an offspring is through sperm retrieval with intracytoplasmic sperm injection (ICSI). Soon-to-be parents should be informed about the potential consequences of this approach because of the probability of vertical transmission of the chromosomal abnormality to their offspring. The genetic risk in the offspring of 47,XXY patients remains unknown. Tachdjian et al. reported on a twin pregnancy delivering two normal karyotype neonates after the ICSI of a patient with non-mosaic KS [52]. Men with sex chromosomal abnormalities have a low or no increased risk of producing offspring with the same abnormalities after ICSI. They are known to have a similar risk as those of men with normal karyotypes [53, 54].

Overall, data have shown that offspring born from KS men do not necessarily have a higher risk of aneuploidies [51]. However, pre-implantation genetic diagnosis (PGD) is a highly logical preventive measure concerning KS. Unfortunately, PGD is not readily available in all fertility centres, especially in developing countries. In addition, PGD is not universally accepted worldwide due to religious or cultural concerns. Using PGD for gender determination, Staessen et al. compared a total of 113 embryos from couples with KS with 758 embryos from control couples [55]. The authors reported a significant decline in the normal embryo rate for couples with KS compared to controls (54.0% vs 77.2%, $p < 0.05$). The authors recommended the use of ICSI in combination with PGD.

In 159 non-mosaic KS men in northeastern China, the mean age of patients with positive sperm retrieval was significantly lower than that of men with negative findings (26.27 ± 3.34 vs 28.44 ± 4.87, $p = 0.032$) [56]. The authors of this study recommended that these men should undergo medical treatment during transition into adulthood, especially if aspiring to be fertile in the future.

Genetic counselling should be reassuring to the couple, particularly to KS men. This counselling should focus on early diagnosis and correct timing of treatment; more importantly, the main goal is to improve the quality of life of all KS men.

ICSI Outcomes in Klinefelter Syndrome

Before the onset of ICSI, fertility for KS men seemed to be a hopeless case. The use of ICSI is a major breakthrough in the treatment of male infertility, particularly in men with KS. A number of studies have extensively described the use of ICSI in patients with KS.

Testicular or rarely ejaculated sperm can be used to achieve paternity in patients with KS through ICSI. Although KS men may present with azoospermia, some still have spermatozoa in their ejaculate. Some studies have discussed the theory of testicular function decline in KS patients with progressive loss of spermatogenic cells and hyalinization of the seminiferous tubules during adolescence [57]. Based on this, the cryopreservation of ejaculated or testicular spermatozoa was proposed as a

method for fertility preservation in KS adolescents. This approach carries several challenges, including ethical concerns about obtaining semen samples from adolescents by masturbation or vibratory stimulation or subjecting them to testicular sperm extraction. Therefore, proper counselling of the patient and his parents is needed before offering this modality. Another main limitation of this approach is that most KS cases are usually diagnosed later in life when they seek fertility, and testicular insult has already occurred.

Ni et al. reported the results of 12 non-mosaic KS patients and 1 mosaic KS patient who underwent 13 ICSI PGD cycles using ejaculated sperm [58]. Fourteen (mean 1.47 ± 0.75) embryos were implanted, resulting in 11 live births. The authors likewise compared the pregnancy outcomes following ICSI with and without PGD using ejaculated sperm. No significant difference was noted in implantation rate (57.69% vs 39.29%, $p = 1.00$) and clinical pregnancy rate (80.00% vs 72.73%, $p = 1.00$) following ICSI with and without PGD. In a recent literature review by Yu et al., a total of 12 mosaic KS patients whose ejaculated sperm were used for ICSI were analysed in 11 reports [59]. A fertilization rate of 80.9% and a live birth rate per transfer of 71.4% were identified. They noted that the risk of transmission of chromosomal aneuploidy using this procedure is very low. Kitamura et al. reported 4 cases out of 52 non-mosaic 47,XXY KS patients having spermatozoa in their ejaculate [28]. ICSI was performed using ejaculated spermatozoa in three of these patients. However, chemical abortion and spontaneous abortion at 8 weeks resulted from two patients mentioned earlier. Testicular sperm was used in one of the patients, which resulted in the delivery of one normal karyotyped healthy boy. In another case report of a severely oligoasthenoteratospermic 34-year-old man with non-mosaic KS, 11 oocytes underwent ICSI using motile spermatozoa from ejaculated specimens [60]. On the 37th week of gestation, twin infants were delivered with normal 46,XX and 46,XY karyotypes. Cruger et al. reported a 28-year-old 47,XXY KS patient whose ejaculated sperm with nine motile spermatozoa were used on the day of the ICSI procedure [61]. This resulted in a singleton pregnancy with a normal 46,XX female baby. Although successful pregnancies were reported using ejaculated sperm, large prospective studies are lacking to recommend its definite use for ICSI.

Testicular sperm harvested through different retrieval techniques is currently the recommended procedure to acquire sperm for ICSI. In an earlier study, an open excisional technique for patients with non-mosaic KS was performed to retrieve testicular sperm for ICSI use [62]. Only four out of nine patients with spermatozoa were successfully retrieved. One patient had a biochemical pregnancy; however, no delivery was noted using this technique. Fine-needle aspiration was also used to obtain testicular sperm for KS patients. In a study by Reubinoff et al., sperm retrieval was successful in four out of seven patients with non-mosaic KS [63]. The authors reported successful pregnancy and delivery using this technique of sperm retrieval, but they considered it to be experimental.

It was not until 1999 that Dr. Peter Schlegel revolutionized the technique of testicular sperm retrieval by introducing microdissection TESE (micro TESE) [64]. This technique has shown significant differences in overall SRR when compared to other techniques in men with NOA, including KS [65].

Majzoub et al. compared the sperm retrieval outcomes between convention TESE ($n = 23$) and micro TESE ($n = 20$) in 43 patients with non-mosaic KS [66]. Overall, 13.9% of testicular spermatozoa were successfully retrieved. SRR was significantly higher in micro TESE than in conventional TESE (30% vs 0%, $p = 0.006$). In men who underwent micro TESE, SRR was significantly higher in men who received hormonal stimulation prior to the procedure than in men who did not receive any stimulation (37.5% vs 0%). Men who were taking anastrozole had increased SRR compared to those men taking clomiphene citrate plus HCG (27.8% vs 12.5).

Several studies on predictors for successful testicular sperm retrieval for KS have been described. Ozveri et al. reported ten non-mosaic KS patients who underwent micro TESE without hormonal treatment [67]. Motile spermatozoa were found in 66.6% of patients. The fertilization rate was 40% following ICSI; however, only one resulted in successful delivery. In another study of 91 men with non-mosaic KS, men with increased baseline testosterone levels had an increased SRR of 86% [68]. By contrast, men who responded to medication with a resultant testosterone level ≥250 ng/ml likewise had increased SRR (77% vs 55%) compared to men with a lesser value of testosterone. In a prospective study of TESE in young (15–22 years) and adult (>23 years) patients with non-mosaic KS, the younger group had a decreased SRR compared to the adult group; however, no statistical significance was attained (52% vs 62.5%, $p = 0.73$) [69]. In a recent study of 110 NOA men with KS, age, testosterone and FSH levels showed associations with micro TESE outcomes [70]. Seventy per cent of men with testosterone levels greater than 2.95 ng/ml had successful SRR ($p = 0.01$). By contrast, elevated FSH levels ($p = 0.17$) and age more than 35.5 years ($p = 0.012$) showed a negative correlation with the outcomes of micro TESE. In a study by Rohayem et al., a total of 135 patients, including 50 pubertal adolescents (13–19 years) and 85 adult patients (20–61 years) with non-mosaic KS, were examined to identify possible predictive factors for sperm retrieval outcome [71]. The adolescent patient group (15–19 years) had a higher SRR than the adult group (45% vs 31%). However, very young adolescents (13–14 years) had the lowest SRR of 10%. Among the hormonal profiles used in the study, only LH levels were significantly higher in those with successful SRR than in those with lower levels of LH (20.0 ± 5.6 vs 13.3 ± 4.3, $p = 0.002$). Koga et al. compared successful and failed microdissection TESE in 26 azoospermic patients with KS [72]. Successful retrieval was achieved in 50% of the patients. Testicular spermatozoa were retrieved successfully in 94.1% of testes that did not contain sclerotic changes in the seminiferous tubules. By contrast, no testicular spermatozoa were found in the testes with sclerotic seminiferous tubules ($p < 0.0001$).

Despite these findings, there are no definitive predictors for successful TESE. Large prospective studies are needed to arrive at this conclusion. At present, there are no established recommendation guidelines for the optimal timing for sperm retrieval [73].

A recent systematic review and meta-analysis on 37 trials comprising 1248 men with KS determined the sperm recovery and ICSI outcomes in this set of patients [74]. They reported an overall SRR per TESE cycle of 44%. Micro TESE had a higher SRR than conventional TESE; however, no significant difference was noted

(45% vs 43%, $p = 0.65$). A meta-regression analysis revealed that other parameters, such as age, testis volume, FSH, LH and testosterone, did not predict successful sperm retrieval. Patients less than 20 years old showed no difference in SRR compared to other patients who were older than them (43% vs 43%, $p = 0.95$). Likewise, bilateral testicular sperm recovery did not show any significant difference in SRR when compared to the unilateral approach (51% vs 44%, $p = 0.34$). The cumulative pregnancy rate and live birth rate showed similar results (43% per ICSI cycle). High FSH levels showed a negative association with LBR per ICSI cycle ($p = 0.06$). No significant difference was noted in the pregnancy rate per ICSI cycle (39% vs 36%, $p = 0.76$) and LBR per ICSI cycle (39% vs 29%, $p = 0.38$) when using fresh sperm compared to cryopreserved sperm. A limited abortion rate of 15% was reported. However, several limitations and possible sources of bias were reported by the authors. A larger trial is needed to reach a definitive conclusion for these patients.

Conclusion

KS is the most frequent X chromosome aberration causing hypogonadism and male infertility. The variants of KS share the same features of hypergonadotropic hypogonadism but differ in phenotypic characteristics. Adolescent and adult patients with KS have varied presentations. Sperm retrieval through micro TESE provides a superior reproductive outcome compared to other techniques. ICSI is a safe procedure with a minimal probability of transmitting sex chromosomal abnormalities. Genetic counselling should be performed through a multidisciplinary approach focusing on the quality of life and overall health status of KS men.

Review Criteria
A thorough search of medical literature was performed on Klinefelter syndrome using the following search engines: PubMed, Google Scholar, MEDLINE and ScienceDirect. The keywords “Klinefelter Syndrome”, “variants”, “testicular sperm extraction (TESE)”, “intracytoplasmic sperm injection (ICSI)”, “male infertility”, “spermatogenesis”, “genetic counselling” and “sperm retrieval” were used for study identification and data extraction.

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Chapter 12
Chromosomal Translocations and Inversion in Male Infertility

Kareim Khalafalla, Pallav Sengupta, Mohamed Arafa, Ahmad Majzoub, and Haitham Elbardisi

Key Points
- Chromosomal abnormalities in functional genes for testicular development and spermatogenesis may lead to male infertility.
- Two most common structural chromosomal abnormalities involved in idiopathic male infertility are chromosomal translocation and inversion.
- Chromosomal translocation refers to the transfer of chromosomal part to another chromosome.
- Chromosomal inversion occurs when a part of the chromosome breaks off, rotates 180 degrees, and reattaches to the same chromosome.

Case 1

A 28-year-old male, without any significant past medical and surgical reports, was presented to the infertility clinic with a 2-year history of primary infertility. His wife is 27 years old and is healthy. His general physical examinations showed that he is normal for an androgenized male. Genital examinations revealed normal testes and epididymis with regard to size and consistency. Initial hormonal profile showed normal levels for FSH, LH, and testosterone. Semen analysis following WHO

K. Khalafalla
Department of Urology, Hamad General Hospital, Doha, Qatar

P. Sengupta
Department of Physiology, Faculty of Medicine, Bioscience and Nursing, MAHSA University, Jenjarom, Selangor, Malaysia

M. Arafa (✉) · A. Majzoub · H. Elbardisi
Department of Urology, Hamad Medical Corporation, Doha, Qatar

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(2010) criteria showed azoospermia with normal volume and positive fructose in semen. Cytogenic analysis was performed revealing a genetic chromosomal abnormality Robertsonian translocation (45XY, der(15,21)(q10,q10). The husband underwent a micro-testicular sperm extraction (TESE), and sperms were detected, and the wife underwent ovarian stimulation followed by ovum pickup. All embryos were tested by preimplantation genetic diagnosis (PGD), and only one out of five embryos was balanced and transferred which resulted in a healthy baby.

Case 2

A healthy 29-year-old male and his 27-year-old wife, who are married for 6 years, reported a history of multiple miscarriages. They had no medical and surgical history and no risk factors for infertility. Physical examinations for the couple did not show any abnormalities. Hormonal profile for the male partner was within the normal range. Semen analysis of two separate samples showed oligoasthenoteratospermia according to the WHO (2010) criteria. Karyotyping revealed the presence of chromosomal translocation in the form of reciprocal translocation (46XY, t(2;4)(P11.2;q31.3). No Y chromosome microdeletion was detected. During counseling, they opted to go for donor sperm insemination.

Case 3

A 40-year-old man was referred to the clinic for primary infertility. His medical and surgical history were insignificant. He has reported to be married recently and had a healthy 30-year-old wife. No family history of frequent abortions or infertility was reported. Physical exam was normal with regard to his general physique, testes size, intact vas deferens, and normal secondary sexual characters. Semen analysis of two separate samples showed azoospermia, and hormonal profile was within normal range. Cytogenic analysis showed the presence of pericentric inversion in chromosome 9 (p11q12) and no microdeletions in the AZF region. The husband underwent micro-TESE, and the wife underwent ovarian hyperstimulation followed by ovum pickup, and all embryos were tested with preimplantation genetic profiling (PGP). However, no embryo transfer was done because all were unbalanced.

Introduction and Definition

Chromosomal abnormalities are one of the prime contributors among the several idiopathic causes of numerous diseases, including male infertility [1]. These are basically anomalies or mutations (deletions or insertions) of the chromosomal DNA [2].

Spermatogenesis and normal testicular development depend on a large number of functional genes [3]. Most of these were believed to be on the male specific Y chromosome such as SRY, AZF, DAZ, USPY, TSPY, DFFRY, CREM, UTY, and MIS. But with time it has been proven that a great portion of them is also present on different autosomes such as SOX9, WT1, FSHR, etc. [4–7] Any aberrations in these genes can cause spermatogenic arrest and testicular dysgenesis which may end up with male infertility. This was postulated almost more than 30 years ago, but it became more evident with time [8].

Types of Chromosomal Anomalies

Chromosomal aberrations can be divided into numerical or structural abnormalities based on their types [9]. This chapter will review two of the most sophisticated structural chromosomal abnormalities, translocation and inversion. Chromosomal translocation is defined as the transfer of a part of the chromosome to another chromosome. The different types depend on the area that is being transferred, whether it's a portion or a complete chromosome and whether it is exchanged or completely transferred [10]. The major two types of translocations are reciprocal and nonreciprocal or Robertsonian translocations [10].

Reciprocal translocation is the exchange of chromosomal material between the arms of two heterologous chromosomes, thus changing the order, but usually not the amount of genetic material [9] (Fig. 12.1). Though any chromosome can display reciprocal translocations, it is most often observed in chromosomes 12, 22, and Y, because of their relative lengths [11]. While unbalanced reciprocal translocations

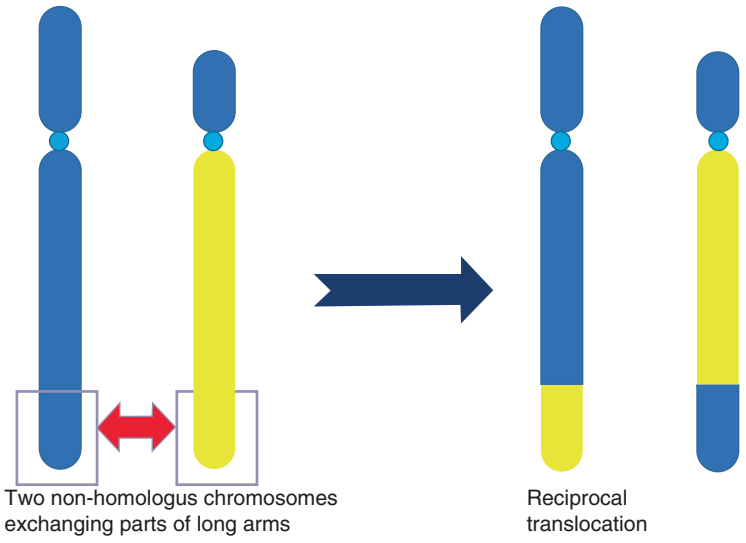

Fig. 12.1 Reciprocal translocation

are usually associated with mental retardation [12, 13] and physical problems [14], balanced forms usually do not have any phenotypic effect on carriers, except that they may show variable semen abnormalities ranging from oligozoospermia to azoospermia or are at an increased risk of producing unbalanced gametes [15, 16]. The mechanism by which reciprocal translocations cause oligozoospermia is not yet elucidated. Studies have suggested that genes critical to spermatogenesis could be disturbed during breakage and reunion of exchanged material (Table 12.1) [17]. On

Table 12.1 Reported reciprocal translocations associated with male infertility

Autosome translocation	*Semen characters*	*Ref*
46XY, t(7;16)(q21.2; p13.3), inv(9)(p11q13).	Sperm count ranging from 2 × 10^6/mL to 5 × 10^6/mL, sperm hypomotility (A + B = 35%), and abnormal sperm morphology. Sperm head defect was 98%	[17]
46XY t(9;13;14) (p22;q21.2;p13)	Azoospermia on multiple semen analysis	[61]
46XY, t(10;15) (q26;12)	Sperm count 42 × 10^6/mL, sperm motility very poor 6%, abnormal sperm morphology reaching 100% specifically sperm head defects	[62]
46, XY, t(18;21) (p11;q21)	Severe oligozoospermia (2–3 sperms found in HPF after centrifugation)	[63]
46 XY, t(11;22) (q23;q11)	Sperm count of 110 × 10^6/mL, sperm motility 5%	[64]
46 XY, t(3;7) (q25;q22)	Low-volume azoospermia	[65]
46XY, t(2;9)(p21;p22)	Azoospermia	[65]
46 XY, t(2;4) (p11;q31.3)	Sperm count 2.3 × 10^6/mL, sperm motility 5%, abnormal sperm morphology 77%	[65]
46 XY, t(11;22) (p10;q10)	Sperm count 2.6 × 10^6/mL, sperm motility 4%, abnormal sperm morphology 94%	[65]
46 XY t(11;19) (p10:p10)	Azoospermia	[65]
46 XY t(5;6) (p10;q10)	Azoospermia	[65]
Genosome translocation	*Semen characters*	*Ref*
45; X; dic(Y;13)(p1?;p12).ish dic (Y;13) (p11:32; p12)	Severe oligozoospermia	[66]
46,X,t(Y;16) (p11;q11)	Azoospermia	[42]
46,X,t(Y;16) (q11;q13)	Oligozoospermia	[67]
46,X,t(Y;16) (q11;p13)	Azoospermia	[68]
46,X,t(Y;16) (q12;q11-12)	Azoospermia	[69]
46,X, t(Y;16) (q11.21;q24)	Oligozoospermia	[70]
46,X,t(Y;16) (q12;q13)	Azoospermia	[30]
46,X,t(Y;13)(q12;p11.2)	Sperm count 0.1 × 10^6/mL	[71]
46, Y, t(X;11) (q26;q21)	Azoospermia on multiple samples	[72]
46, Y, t(X;18) (q22.3;q23)	Sperm motility <25%, abnormal spermatozoa 99%	[41]
46XY, t(Y;10) (q11.2; q24)	Sperm count 1.2 × 10^6/mL, sperm motility 0%, abnormal sperm morphology 88%	[65]
Recommendations		*GR*
Obtain standard karyotype analysis in all men with damaged spermatogenesis (spermatozoa <10 million/mL) who are seeking fertility treatment by IVF		B
Provide genetic counseling in all couples with a genetic abnormality found in clinical or genetic investigation and in patients who carry a (potential) inheritable disease		A

the contrary, the translocated chromosomes form a pairing cross during meiosis, hindering the meiotic process and leading to spermatogenic arrest [18, 19] which can also cause oligozoospermia.

Robertsonian (nonreciprocal) translocation is a structural chromosomal aberration that occurs due to the centromeric fusion of acrocentric chromosomes which have centromeres located near an end [1] (Fig. 12.2). Humans normally have five pairs of acrocentric chromosomes, namely, chromosome number 13, 14, 15, 21, and 22 [20]. The resulting balanced karyotype shows 45 chromosomes, including the translocated one, which is made up of the long arms of two acrocentric chromosomes [21]. Carriers of balanced translocations are phenotypically normal, but they are at risk of infertility, repeated miscarriages and offspring with unbalanced karyotypes [22]. Individuals carrying each of the ten possible nonhomologous Robertsonian translocations of the five human acrocentric chromosomes have been reported, but the two combinations (13:14) and (14:21) have been observed at a greater frequency than the rest with frequencies of 73% and 10%, respectively [9].

Apart from these, genetic inversion has been considered as one of the structural chromosomal abnormalities that is commonly associated with translocation abnormalities. In genetic inversion, a part of the chromosome breaks off, rotates 180 degrees, and reattaches to the same chromosome (Fig. 12.3). It's a matter of rearrangement rather than genetic loss [9]. Inversions are of two types, pericentric and paracentric, depending on whether it contains a centromere or not. Paracentric does not include a centromere and breaks occur in one arm of a chromosome, while peri-

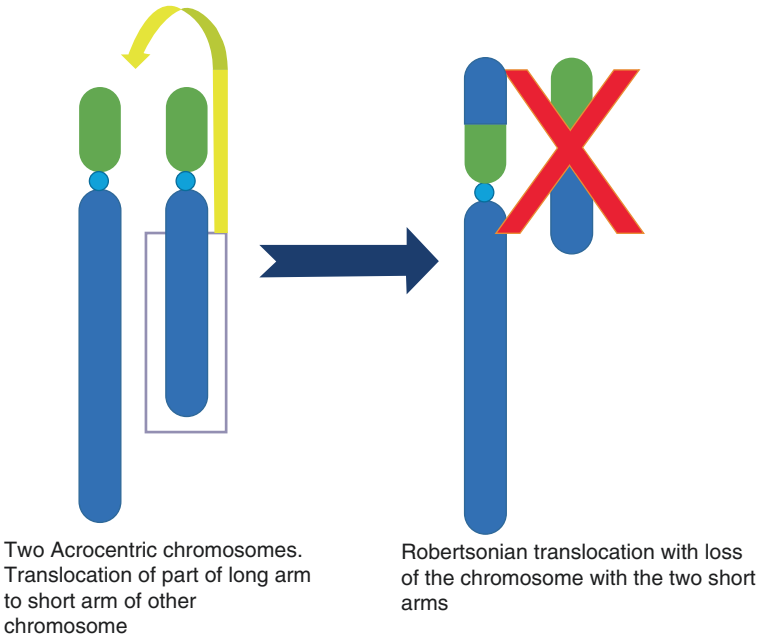

Fig. 12.2 Robertsonian translocation

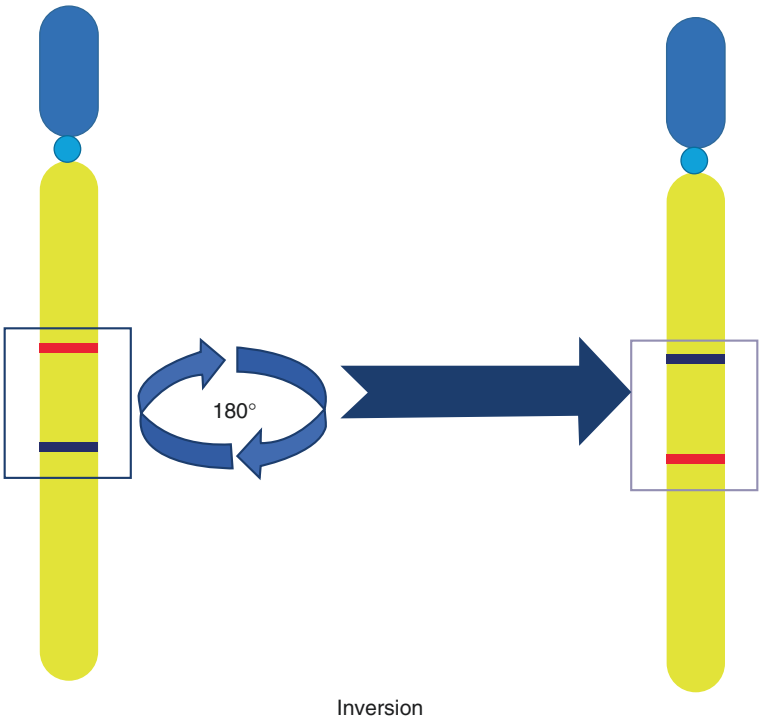

Fig. 12.3 Chromosomal inversion

centric inversion includes a centromere and break occurs in both the chromosome arms [23, 24]. Inversion causes abnormalities in carriers when it occurs in heterozygous individuals, while they may pass unnoticed with a normal phenotypic character in homozygous balanced cases. Chromosome 9 inversion is considered the most common type seen in humans, especially at inv(9)(p12q13) [25].

Incidence

The etiology of male infertility has been strongly linked to genetic and chromosomal aberrations with a wide varying frequencies from as low as 2.2% and high up to 15% [26]. Robertsonian translocation has been reported in literature in different case reports and studies for infertile men between 0.7% and 3%. [22, 27] They are recognized as the most common structural rearrangements in general population, occurring in 1.23% of births [28]. Reciprocal translocation has a much less incidence in azoospermic and oligozoospermic men reaching up to 0.9% with azoospermic patients having a higher upper hand incidence. [29, 30] Pericentric Inversions in azoospermic and oligospermic men complaining of infertility and seeking in vitro

fertilization (IVF) treatments range from 0% to 0.3% [31]. Chromosome 9 inversions are seen in 1% to 4% depending on the population studied [32, 33].

Mechanisms

Reciprocal translocations have many reported mechanisms of occurrence and their impacts on male infertility [34, 35]. One of these theories is related to spermatogenetic genes which are affected by chromosomal aberrations, causing their inactivation or deletion. In the process of normal spermatogenesis, it has been well established that protamines replace histones during the chromatin packaging in the sperm head. Alterations of genes coding for protamines and transition proteins, like PRMI, PRM2, and TNP2, can affect sperm differentiation, motility, and function explaining the cause of infertility [36, 37]. Another theory is that the translocated chromosomes may form a centromeric association with the XY bivalent at the pachytene stage of meiosis-I, which may interfere with X chromosome inactivation. This leads to a lethal gene dosage effect on the germ cells resulting in spermatogenic arrest [19, 38].

Reciprocal translocations can also affect infertility being inherited from the female partner causing ovarian failure and gonadal dysfunction [39]. This is done mainly through X chromosome translocation. It has been reported that X chromosome activation or inactivation, in combination with expression of different genes, can occur as translocation of genetic material from the X chromosome. Several regions in the X chromosome can display translocation, for instance, X 1–4, 6–9, 11, 12, 14, 15, 17, 19, 21, and 22 [40, 41].

With regard to Y chromosome translocation, it has been postulated that the Yp11 may contain unknown regulatory genes for spermatogenesis which are affected by translocation, causing infertility [42]. Previous studies handling with Y chromosome translocation suggested a theory of meiosis disturbance with chromosomes 1,9,16 between some heterochromatin regions and interphase nucleolus [43, 44].

Clinical significance of inversion is related to the consequence of each chromosomal rearrangement separately. Different hypotheses have been postulated but none fully confirmed to be the key cause for infertility, which can be translocation as well as affecting the meiotic pachytene stage, resulting in spermatogenic arrest and germ cell apoptosis [45, 46]. Another opinion is that the process of inversion yields a lethal product with a reduced recombinant frequency hindering the normal process of spermatogenesis especially if it occurs at breakpoints of important genes in spermatogenesis [47].

Clinical Presentations

Patients with chromosomal translocations or inversions usually are subfertile or infertile. They may have unexplained abortions and IVF failures. Semen analysis for such patients usually shows azoospermia or severe oligoasthenospermia, or a

low semen profile. Wife's evaluation is usually insignificant, and male hormonal profile together with the physical examination is also usually normal or elevated FSH with borderline to low testosterone. On further investigations including testes biopsy and karyotype analysis, results of maturation arrest and spermatogenesis failure can be seen, and genetic analysis possibly shows different aberrant chromosomes.

According to the American Urological Association (AUA), recommendations for karyotyping and genetic counseling should be offered to all patients with nonobstructive azoospermia and severe oligospermia ($<5 \times 10^6$ sperms/mL). Similarly, the European Association of Urology (EAU) recommendations are based on the frequencies of chromosomal aberrations in patients with different sperm concentration, and karyotype analysis is indicated in men with azoospermia or oligozoospermia (spermatozoa $<10 \times 10^6$/mL).

Management

Despite spontaneous pregnancies being reported in such cases, intracytoplasmic sperm injection (ICSI) and PGD are considered the hallmark of management of such cases. With the advent of reproductive techniques and breakthrough in testicular sperm extraction, such cases of severe male factor infertility with nonobstructive azoospermia are given the chance of fathering their own children. The pregnancy rate after assisted reproductive technique (ART), in cases with chromosomal translocation, is reported in very few studies. In Robertsonian translocation, the pregnancy rate has been reported from 20% to 25% in carriers undergoing ART [48]. To the best of our knowledge, no studies have reported pregnancy rate in reciprocal translocation carriers undergoing ART. The sperm retrieval rate with testicular sperm extraction in azoospermia cases with translocation was not reported before in the literature. The sperm retrieval can be done by TESA (testicular sperm aspiration), and if no sperm was found, TESE (testicular sperm extraction) can be performed, either by conventional or microsurgical method.

Preimplantation Genetic Screening (PGS) or Preimplantation Genetic Diagnosis (PGD)

Preimplantation genetic screening (PGS) is defined as the evaluation and screening of chromosomally normal individuals searching for chromosomal numerical abnormalities, either too many or too few chromosomes. This is mainly offered for those with repeated implantation failures, abortions, advanced maternal age, and severe male factor. On the contrary, preimplantation genetic diagnosis is the evaluation for specific genetic abnormalities from embryos of previously documented parents having genetic abnormalities such as single mutation, translocations, and

genetic diseases [49]. It includes a series of activities to select a completely healthy embryo for transfer. This process includes taking a biopsy, preparing the genetic material with DNA isolation and amplification, followed by analysis and result preparation [50].

There are numerous different methods that have evolved over the years to conduct PGD/PGS. The key technologies involve fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), single-nucleotide polymorphism (SNP) analysis, and very recently next-generation sequencing (NGS) which is considered as the state-of-the-art method with the highest accuracy and efficiency [50].

Reproductive Counseling

Many couples are not acquainted with their genetic abnormalities specially if they had no implications on their life and what their presence means for a pregnancy; thus, genetic counseling can ensure that the couples understand the implications of these conditions. In addition, the couple should receive information about all their reproductive options, including the option of PGS/PGD combined with IVF and ICSI, conceiving naturally, use of donor sperm in combination with IVF/ICSI, or intrauterine insemination (IUI) and adoption. For some couples, the increased risk of chromosomal abnormalities in children is not a risk that they are willing to take; thus, the option of using donor sperm or adopting might be more appealing. In a retrospective study of 407 couples with male factor infertility who discontinued treatment without conceiving, 11% pursued adoption, and 1% of couples used donor sperm. The role of the genetic counselor is to inform the patients of all of their options so that couples can make informed decisions about future reproductive attempts [51–53].

Genetic counseling starts by obtaining a three-generation pedigree for possible family history of infertility, recurrent pregnancy loss, birth defects, disability, and genetic disease. A thorough discussion with the couples explaining that all pregnancies have 3% risk for birth defects and intellectual disability inconsiderate of maternal age or family history [54, 55].

PGD is done to allow translocation carriers to conceive balanced offspring and decrease the risk of miscarriages. It is well known that translocation carriers produce many unbalanced embryos, and PGD is their only chance of conceiving biological offspring only if the woman responds to ovarian hyperstimulation by producing many oocytes to increase the chance of having a balanced embryo [56, 57].

All couples are thus evaluated and counseled by infertility specialists and medical geneticists. The procedure and limitations of PGD must be explained to the couples including the risk of misdiagnosis attributable to embryonic mosaicism and the 1% to 2% technical error rate of the fluorescence in situ hybridization (FISH) procedure used in PGD [58–60].

Conclusion

The present chapter has discussed how chromosomal translocations or inversions are associated with male subfertility or infertility. Patients with genetic anomalies often suffer from unexplained abortions and IVF failures. Men with chromosomal abnormalities mostly are diagnosed with azoospermia, severe oligoasthenospermia, or a low semen profile. It is required to acquaint the couples regarding their genetic abnormalities even if they cause no implications on their present life. This is vital because chromosomal abnormalities in male may also impact upon pregnancy outcome of their partners; thus, genetic counseling can ensure that the couples understand the implications of these conditions.

Review Criteria
An extensive literature search has been performed to find the relationship between chromosomal translocations, inversions, and male infertility using search engines such as Science Direct, OVID, Google Scholar, PubMed, and MEDLINE. The overall strategy for study identification and data extraction was based on the following keywords: "chromosomal abnormalities," "chromosomal inversion," "chromosomal translocation," "male infertility," and "semen parameters." Articles published languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book chapter citations provide conceptual content only.

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Chapter 13
Genetics of Vas Aplasia

Rahul Krishnaji Gajbhiye, Shagufta Khan, and Rupin Shah

Key Points

- Congenital bilateral absence of the vas deferens (CBAVD) is a common cause of obstructive azoospermia. CBAVD is seen in most men with cystic fibrosis, while isolated CBAVD is frequently associated with mutations in the CFTR gene.
- CBAVD associated with renal anomalies is often due to causes other than CFTR mutations.
- There are over 2000 mutations of the CFTR gene, and there is considerable ethnic variation. Population-specific mutation panels and mandatory guidelines are required for Asian and African countries.
- Unilateral absence of the vas (CBAVD) may be due to CFTR mutations or may be due to other causes when it is associated with ipsilateral renal agenesis.
- In CBAVD, spermatogenesis is essentially normal, and pregnancy can be obtained through ICSI using sperm aspirated from the epididymis or testes.

R. K. Gajbhiye · S. Khan
Department of Clinical Research, ICMR-National Institute for Research in Reproductive Health, Mumbai, Maharashtra, India

R. Shah (✉)
Department of Andrology, Lilavati Hospital and Research Centre, Mumbai, Maharashtra, India

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Clinical Scenarios

Case 1

VN (29 years old) and SN (25 years old), an unrelated South Indian couple, presented with primary infertility of 2 years duration. There was no family or personal history of cystic fibrosis. The semen analysis showed low volume (<1 ml), azoospermia, and absence of fructose. On examination, testicular volume was normal, and bilateral vasa deferentia were not palpable. Hormone profile was normal. Transrectal ultrasound (TRUS) revealed congenital bilateral absence of seminal vesicles (CASV) with small Mullerian duct cyst. Ultrasound (USG) of the abdomen showed bilateral normal kidneys. After obtaining informed consent and providing counseling, blood samples of both VN and SN were collected and processed for *CFTR* gene mutations screening. Direct DNA sequencing of essential promoter, entire coding regions, and splice sites of 27 exons of the *CFTR* gene were carried out in male and female partner. VN was found to carry two mutations: a common mutation associated with classical cystic fibrosis (CF) c.1521_1523delCTT (F508del) and a novel *CFTR* mutation L578I. There was no family history of CF or past medical history of any respiratory, pancreatic, or gastrointestinal symptoms suggestive of CF. SN was found to be heterozygous carrier of A1285V, a novel *CFTR* mutation detected in North Indian men with CBAVD [1]. The couple was counseled and advised prenatal genetic diagnosis in view of the risk of having a child with classic CF or CFTR-related disorders such as CBAVD.

Case 2

RS (28 year old) and PS (26 years old), healthy, unrelated, Gujarati Indian couple married since one and half years, were referred for primary infertility. Semen analysis showed azoospermia with absent fructose and volume < 1 ml. On scrotal examination, testes were normal, only the head of the epididymis was palpable, and bilateral vasa were found to be absent. TRUS confirmed CASV. Abdominal USG showed absence of right kidney with left ectopic kidney in pelvis on left side of urinary bladder with mild compensatory hypertrophy. RS was diagnosed as CBAVD with unilateral renal agenesis (URA). Direct DNA sequencing of essential promoter, entire coding regions, and splice sites of 27 exons of the *CFTR* gene could not detect CF or CBAVD causing mutations except 3 previously reported potential regulatory coding *CFTR* gene variants (AGA haplotype) c.1540G > A V470 M (heterozygous), c.2694 T > G T854 T (heterozygous), c.4521G > A Q1463Q (homozygous), and TG12-5T/TG11-7T (heterozygous). The female partner was not a CF carrier. Genetic counseling was provided to the couple. Percutaneous epididymal sperm aspiration (PESA) revealed good-quality motile sperm. The couple underwent one cycle of ICSI; however, there was no pregnancy.

Case 3

SS (28 year old) and RS (26 year old), healthy, unrelated Jain couple, presented with 3 years of infertility. Semen analysis indicated low volume, azoospermia with absence of fructose. Scrotal examination revealed a thick, palpable right vas deferens and a non-palpable left vas deferens. TRUS confirmed the absence of left vas deferens and left seminal vesicle. Right seminal vesicle was markedly dilated, filled with intraprostatic fluid. The right terminal vas was not identified. USG abdomen showed absence of the left kidney with compensatory hypertrophy of the right kidney. SS was diagnosed as congenital unilateral absence of vas deferens with unilateral renal agenesis (CUAVD-URA). Blood samples of both SS and RS were collected and processed for CFTR mutation screening. Direct sequencing of essential promoter, entire coding regions, and splice sites of 27 exons of the CFTR gene could not detect CF or CBAVD causing mutations except c.1210–12[5] [5T] variant. The female partner was not a CF carrier. After providing counseling, the couple underwent two cycles of ICSI resulting into live birth of a female child in the second ICSI cycle.

Vas Aplasia

Congenital bilateral absence of the vas deferens (CBAVD) is associated with normal spermatogenesis and obstructive azoospermia, involving a complete or partial defect of the Wolffian duct derivatives [2]. CBAVD affects 2–3% of all male infertility cases and is responsible for 25% cases of obstructive azoospermia [3–5]. The etiology of CBAVD is not completely understood; however, there is a well-established linkage between CBAVD, cystic fibrosis, and CFTR gene mutations [3, 6]. The human male internal genitalia originate from the paired Wolffian ducts (WDs), which in the male embryo are stabilized by testosterone. The ducts develop into separate but connecting organs, the epididymis, vas deferens, and seminal vesicles. During development at 6 weeks of embryonic growth, the WD opens to the urogenital sinus at a site adjacent to the ureteral orifice (Fig. 13.1a). At 7–8 weeks, there are discrete differences in the WD position along the mediolateral axis as well as in the morphology of the urogenital sinus (Fig. 13.1b). At 8–9 weeks, the bilateral upper angles of the urogenital sinus start upward growth toward the umbilicus. During the ninth week depending on development of smooth muscles in the bladder as well as rhabdosphincter muscles of the urethra, the descent of the vas deferens becomes evident (Fig. 13.1c). At 10–11 weeks, a radical ascending development of bladder smooth muscles as well as a developing prostate accelerates the descent of the vas [7] (Fig. 13.1d). The effects of the CFTR mutations on the WD may occur after the ninth week of development causing CF- or CFTR-related disorders (CFTR-RD). In the embryo, the penetration of the metanephrogenic blastema by the ureteric bud induces the development of the kidney. Any interruption of this process

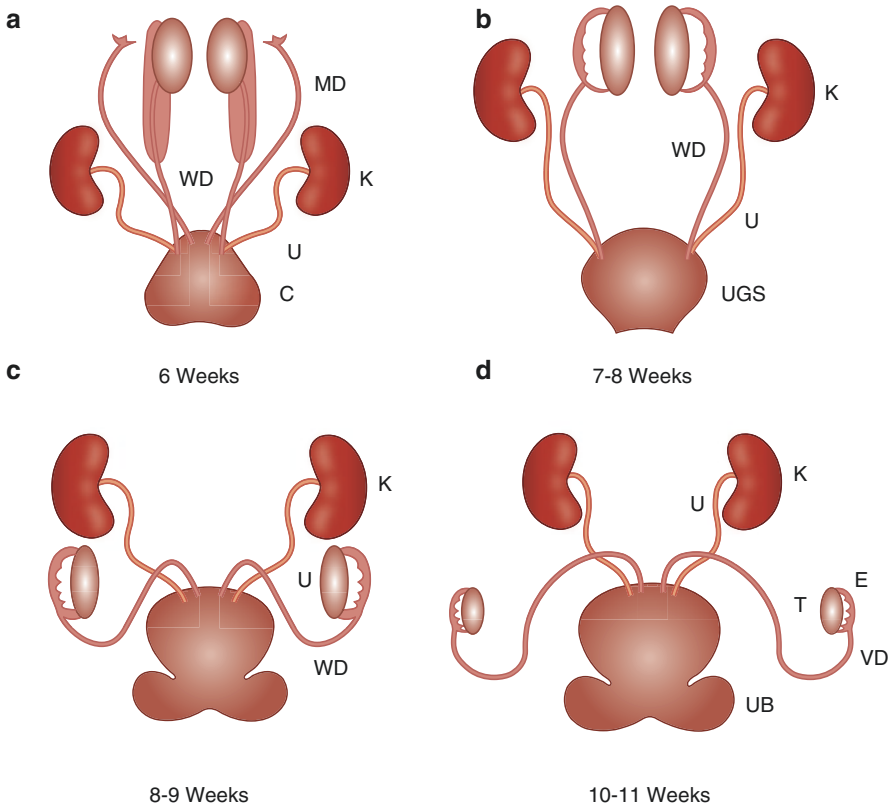

Fig. 13.1 Embryogenesis of male genitalia. (**a**) 6 weeks: *WD* Wolffian duct, *MD* Müllerian duct, *K* kidney, *U* ureter, *C* cloaca. (**b**) 7–8 weeks: *WD* Wolffian duct, *K* kidney, *U* ureter, *UGS* urogenital sinus. (**c**) 8–9 weeks: *WD* Wolffian duct, *K* kidney, *U* ureter. (**d**) 10–11 weeks: *VD* vas deferens, *E* epididymis, *T* testis, *K* kidney, *U* ureter, *UB* urinary bladder

before the complete separation of the WD and ureteric bud can result in renal agenesis (URA) and CUAVD, whereas interruption in the development of the WD after the separation may lead to an isolated CUAVD [7].

Genetic Abnormalities in Vas Aplasia

The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene was first reported as causative factor in cystic fibrosis (CF) [8]. Almost 95% of CF men have CBAVD [6]. CBAVD reported in infertile but otherwise healthy men (without CF) is known as isolated CBAVD and is now classified as CFTR-related disorders [3]. There is a different spectrum of CFTR mutations in CBAVD and classical CF [9]. However, majority of CBAVD men (88%) have one severe and one mild CFTR

mutation or two mild CFTR mutations (12%) but never carry two severe CFTR mutations [3, 10]. The *CFTR* gene is located on the long arm of chromosome 7q31.2 and contains 27 coding exons that spread over 230 kb. Its 6.5-kb mRNA encodes a 1480-amino acid protein that regulates chloride channel in a variety of tissues [11, 12]. Any defect in *CFTR* gene contributes to abnormal electrolyte transport in the epithelial cells of the respiratory tract, the pancreas, the intestine, the vas deferens, the hepatobiliary system, and the sweat glands [13]. The CFTR mutations have been traditionally classified into six classes based on clinical severity of CFTR mutations (Table 13.1). Class I and II mutations are common, classes III and IV are uncommon (1–5% of CF mutations), and class V and VI mutations are very rare (<1% of CF mutations) [14]. CBAVD is typically caused by a residual function CFTR class IV or V mutation, resulting in less than 10% of wild type CFTR function (Fig. 13.2). Recently, De Boeck and Amaral suggested seven categories wherein classes I, II, and III, and VII are defined as severe mutation, while classes IV, V, and VI are associated with mild phenotypes [15].The traditional class I mutations have been divided into class I (stop-codon mutations) and a new class VII mutation wherein there is no mRNA transcription resulting in absence of the CFTR protein similar to the traditional class I mutation; however, it cannot be altered by drug therapy. Marson's

Table 13.1 Classification of CFTR mutations

Class	Molecular defect	Effect on CFTR Protein	Functional CFTR present	Examples
I	Mutations cause premature stop codon to prematurely stop the translation of mRNA	Defective protein synthesis	No	Trp1282X, Arg553X, Gly 542X
II	Mutations cause defective protein processing and impaired trafficking	Misfolded protein not transported to surface	No/reduced	Phe508del, Asn1303Lys, Ala561Glu
III	Mutations result in amino acid substitution disrupting regulation of channel	Reduced or lack of chloride channel opening	No (nonfunctional CFTR present in apical membrane)	Gly551Asp, Ser549Arg, Gly1349Asp
IV	Mutations cause amino acid substitution resulting in a defect in the CFTR structure that forms a pore	Misshaped pore inhibits the flow of chloride ions through the channel	Yes	Arg117His, Arg334Trp, Ala455Glu
V	Missense mutations disrupt mRNA synthesis generating both normal and alternative transcripts	Reduced normal protein synthesis causing lesser protein being transported on the surface	Yes/reduced	3272-26A→G, 3849 + 10 C→T
VI	Mutations increase production of protein at cell surface	Excess CFTR is unstable and degraded by cell machinery	Yes (unstable)	120del23, rescued F508del, N287Y

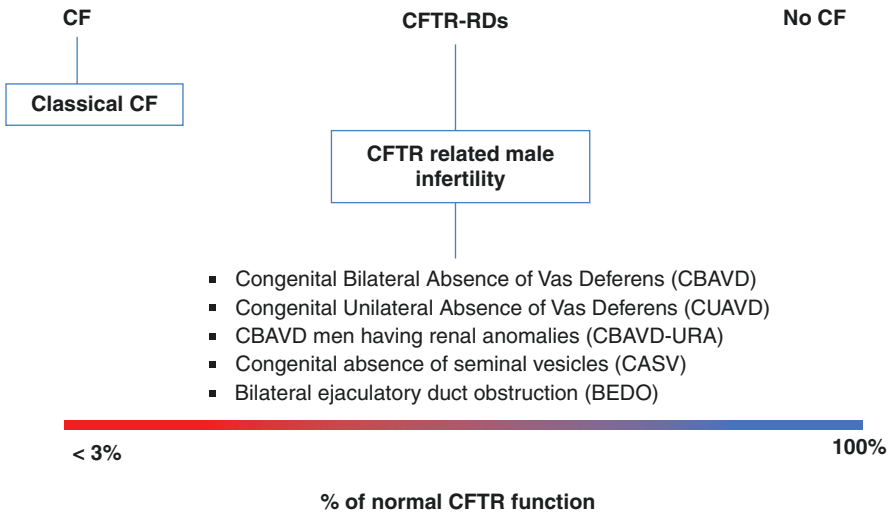

Fig. 13.2 Cystic fibrosis transmembrane conductance regulator (CFTR)-related male infertility

group proposed that CFTR mutation class VII is important and be retained as class IA, which includes mutations with severe phenotype where corrective therapy is unavailable, followed by classes IB to VI [16].

More than 2000 CFTR gene sequence variants have been reported since the discovery of the CFTR gene [17], and there are three different CFTR databases – http://www.genet.sickkids.on.ca/, http://www.umd.be/CFTR, and http://www.cftr2.org/ [18]. F508del is the most common severe mutation detected in CF and CFTR-RDs with 50–80% frequency among Caucasians, while other known mutations occur with a lower frequency (<6%) [3]. Additionally, mild variants in intron 9 (IVS9) with poly thymidine 5, 7, and 9 affect the splicing of exon 10. The TG repeats (TGm) located upstream to the poly T tract in IVS9 and the polymorphism c.1408G>A, p. (Met470Val) (M470V) rs213950 in exon 11 (HGVS) have been implicated in CBAVD [19, 20]. The frequencies of mutations have been reported to vary with different geographical and ethnic variations [3, 5, 21]. Majority (95%) of CF-CBAVD patients have mutations in the CFTR gene [22] as compared to isolated CBAVD, wherein CFTR gene mutations are detected in 60–70% of patients [23]. Failure to detect CFTR abnormality in isolated CBAVD men could be due to limitations of the mutation detection methods or due to etiology other than CFTR gene. The most common CBAVD genotypes reported in European population are the F508del in trans (located in two different chromosomes) with IVS8-5T (28%) and F508del in trans with R117H (6%) [3, 22]. However, there is a variation in frequency of most common CBAVD causing known CFTR mutations [F508del, c.1210–12[5] (5T)] in CBAVD men residing in different geographical regions (Table 13.2). Although there is significant variability in the frequency of F508del

Table 13.2 Ethnic differences in frequency of F508del and 5T mutations in CBAVD men

Country	F508del (%)	5T (%)
Germany [57]	26	13
Spain [29]	18	23
France [10]	22	19
Egypt [58]	2.5	43.7
Turkey [56]	2.9	19.6
Taiwan [59]	0	44.4
Japan [26]	0	29
China [60]	0	46.5
India [24, 58]	11	39.4

mutation in CBAVD men of different ethnic origins, the 5T variant is found to be present at the same or very similar frequency in CBAVD men from Asia and Europe [Indians, 25–39.4% [24, 25]; Japanese, 30% [26]; Turkish, 19.6% [27]; Iran, 25.9% [28]; Spanish, 23% [29]; Portuguese, 27.4% [30]]. This evidence suggests that the 5T variant plays a role in the pathogenesis of CBAVD even in populations considered to have low CF incidence [3].

The identification of large rearrangements and deletions in the CFTR gene of CBAVD patients with absence of mutations has become possible due to improved techniques of mutation analysis [31–33]. Polymorphisms in genes such as Tr2GFB1 (transforming growth factor) and EDNRA (endothelin receptor type A) may increase the penetrance of CBAVD-related mutations [34]. Mutations are usually detected in 80% CBAVD cases; however, failure to detect mutations in the remaining 20% indicates the involvement of genetic etiology other than CFTR gene. Recently, a new pathogenic gene, ADGRG2, encoding the efferent duct and epididymal-specific G protein-coupled receptor with an X-linked inheritance pattern, has been reported in CBAVD patients who were negative for CFTR mutations [2, 35, 36]. A study in a Chinese population indicated differences in the mutations of the promoter region of the CFTR gene as compared to Caucasians. The homozygous c.-966 T >G mutation state had the highest frequency, which reduced the CFTR transcription level [37]. The use of next-generation sequencing (NGS) in evaluating CBAVD men will further improve our understanding of the novel genes that might be involved in the pathogenesis of CBAVD and related phenotypes [38].

Clinical Diagnosis of Vas Aplasia and Associated Subphenotypes

CFTR-related male infertility is subdivided into following subphenotypes:

(a) CBAVD
(b) CUAVD
(c) CBAVD-URA

(d) Congenital absence of seminal vesicles (CASV)
(e) Bilateral ejaculatory duct obstruction (BEDO)

CBAVD

CBAVD is usually detected at adulthood during evaluation of infertility in otherwise asymptomatic males or at the time of a surgical procedure as an incidental finding. In CBAVD men, there is bilateral absence of the vas deferens along with the body and tail of the epididymis and also bilateral or unilateral absence of seminal vesicles. The head of the epididymis is present in all CBAVD cases and has normal function [39]. In some of the CBAVD men, vasa deferentia may be palpable in the scrotum, but during surgical exploration, a fibrous cord or a nonpermeable duct or a blind-ending vas is observed [3]. Isolated CBAVD is now suggested to be a CFTR-related disorder (CFTR-RD) though as per the latest consensus it does not fulfill the diagnostic criteria for CF [3].

CBAVD can be easily diagnosed by a semen analysis showing azoospermia, low seminal volume (<1 ml), low or absent fructose and low semen pH (<6.8) [3], and an impalpable vas deferens on scrotal examination; however, there may be a delay of 4.3 years in correct diagnosis of CBAVD as it can be overlooked by first investigators [40]. Transrectal ultrasonography (TRUS) would usually reveal absent seminal vesicles and ejaculatory ducts, though seminal vesicle-like structures can be observed in 15% of men with vas aplasia and can be a cause of diagnostic confusion. Abdominal and pelvic USG is required to diagnose abnormalities of the upper urinary tract. In majority of CBAVD men, testicular volume and serum gonadotrophins levels are normal, and testicular biopsy shows normal or slightly defective spermatogenesis [40].

CUAVD

CUAVD is a rare entity with a reported incidence of 0.5–1% and is often associated with renal agenesis [41]. Due to the possibility of pregnancy because of the normal function of the other vas deferens, it is likely that the incidence of CUAVD is underestimated [42]. There is a higher incidence of renal anomalies in CUAVD men than in CBAVD men, with an absolute risk increase of 20.1% [43]. Renal anomalies in CUAVD men are reported as malrotation of the solitary kidney, multicystic kidney, ectopic kidney, and horseshoe kidney [44]. CFTR mutations were reported in CUAVD with a lower frequency than in CBAVD [3]. Recently, Klinefelter's syndrome (KS) cases were reported in association with CUAVD harboring CFTR gene mutations including delta F508 in KS-CUAVD cases [45]. Recent meta-analysis demonstrated a fairly high frequency of overall CFTR variants in CUAVD men with very low frequencies of the heterozygous genotypes F508del/5T and F508del/R117H [43]. Additionally, CUAVD men showed increased of 5T risk allele with a 15.5% frequency [43]. These observations suggest the need for detailed physical examination and genetic screening in CUAVD patients.

CBAVD Men Having Renal Anomalies (CBAVD-URA)

A proportion of CBAVD men (11–20%) suffer from concomitant urogenital abnormalities including unilateral renal agenesis [3]. Therefore, CBAVD or CUAVD men should undergo ultrasound examination of the abdomen and pelvis for detection of renal abnormalities. There is limited information on the exact mechanisms involved in the etiology of CBAVD-URA. The role of CFTR gene mutations in this subset of patients is questionable as majority of studies have failed to detect CFTR gene mutations in CBAVD-URA [3, 46]. Genetic factors other than CFTR gene are suggested to be involved in etiology of CBAVD-URA [3].

Congenital Absence of Seminal Vesicles (CASV) and Ejaculatory Duct Obstruction (BEDO)

CBAVD men usually have bilateral or unilateral absence or hypoplasia of seminal vesicles. CFTR gene mutations were detected in infertile men with BEDO and concomitant seminal vesicle anomalies suggesting CFTR gene abnormalities as molecular basis of the genital tract anomalies and the resulting infertility in this subset of obstructive azoospermia [47]. The authors further suggested that BEDO with concomitant seminal vesicle anomalies to be considered as CFTR-associated disorder confined to the male genital tract as there was considerable overlap of CFTR gene mutations in CBAVD and in BEDO with concomitant seminal vesicle anomalies [47].

Infertile men with BEDO are now able to become biological fathers with the support of assisted reproductive technologies [48]; however, such men and their female partners should be provided genetic counseling and CFTR gene mutation analysis to prevent the transmission of genetic abnormalities to the offspring.

CBAVD, Assisted Reproduction, and Genetic Counseling

Although majority of the CBAVD men show normal spermatogenesis on testicular biopsy and sperm from CBAVD men are capable of fertilizing an egg, these men were deprived of biological fatherhood until 1987. Since the caput of epididymis is always present in CBAVD men, recent advances in assisted reproduction technologies as well as sperm retrieval techniques now allow these obstructive azoospermic men to enjoy biological fatherhood. However, once the clinical diagnosis of CBAVD is confirmed, counseling should be offered to the CBAVD male and female partner to screen for CFTR gene mutations because of the high risk of transmitting CFTR mutation(s) to the offspring. Silber and coworkers [49] documented the first pregnancy for a couple in whom the male partner had CBAVD. They utilized the microsurgical epididymal sperm aspiration (MESA) technique and in vitro fertilization in 1988. Since then, other techniques such as PESA (percutaneous epididymal sperm aspiration), FNA (fine-needle aspiration), and TESA (testicular sperm aspiration) have also been used to obtain sperm from men having CBAVD, and the advent of

ICSI (intracytoplasmic sperm injection) dramatically improved fertilization and pregnancy rates [6]. Kamal et al. reported no difference in the rates of fertilization, clinical pregnancy, and miscarriage between CBAVD men (n = 434) and infertile men having other causes of obstructive azoospermia (n = 687) [50]. This study also reported similar rates of fertilization, pregnancy, and miscarriage with use of epididymal spermatozoa and testicular spermatozoa for ICSI [50]. However, another study suggested that CFTR mutations may lead to increased risk of miscarriage and stillbirth and a reduced rate of live birth in CBAVD men compared with non-CBAVD men [51]. Another study in Chinese men found no significant difference in fertilization, implantation, or clinical pregnancy rates between CBAVD and non-CBAVD patients who had PESA followed by ICSI, but there was a significantly lower live birth rate and significantly higher miscarriage rate in CBAVD men as compared to non-CBAVD men. The authors suggested that this increased risk of miscarriage or stillbirth may be associated with the CFTR mutations [23]. Finally, a 10-year ICSI outcomes data analysis evaluating the impact of the quality of testicular spermatogenesis (as determined histopathologically) in CBAVD men suggested that impaired spermatogenesis had a negative impact on early-stage biological outcomes of ICSI [52].

The present American Society for Reproductive Medicine (ASRM), American College of Obstetricians and Gynecologists (ACOG), and American College of Medical Genetics and Genomics (ACMG) recommendation for genetic diagnosis or risk prediction of CBAVD includes either expanded CFTR mutation testing or carrier screening for the 23 most prevalent CFTR mutations [53]. The CFTR mutation panels are well characterized for Caucasians as compared to Asians, Africans, and other populations. The existing CFTR mutation panels were derived from the data of CF patients of Caucasian and Northern European descent and have limited utility for CBAVD men of non-Caucasian origin. Additionally, there are large rearrangements such as exon deletions, insertions, or duplications in 2% of CBAVD men, which are undetected by standard sequencing analysis [54]. The role of modifier genes *TGFβ-1* and *EDNRA* in development of CBAVD has also been reported [34, 55]. Recently, mutations in the *ADGRG2* gene are reported in CFTR-negative CBAVD men [2, 36]. Therefore, the current ASRM, ACOG, and ACMG recommendations for genetic diagnosis or risk prediction of CBAVD have limitations, and there is a need to develop regional guidelines based on the ethnic-specific CFTR mutation screening, especially for the people of Asian and African origin.

Future Perspectives

Evidence suggests that majority of CBAVD is associated with CFTR gene abnormalities; however, there are limitations to the currently available CFTR screening panels. Additionally, new information suggesting genetic involvement other than CFTR needs to be taken into consideration while offering the genetic counseling to the CBAVD men and female partners enrolled for an ART program. There is growing

evidence suggesting similar incidence of CBAVD in Asian, African, and non-European population to that of Caucasians. Therefore, larger studies should be conducted in Asian, African, and non-European CBAVD populations to determine the CFTR mutation spectrum and other genes involved in etiology of CBAVD. Similarly, female CF carrier frequency should also be determined in these populations which were once considered as having low incidence of CF and CFTR-RD. Regulatory guidelines need to be framed and should be strictly implemented, especially for Asian and African populations, on mandatory CFTR screening and genetic counseling before undergoing ART. This can be achieved with the support of the WHO and international NGOs working for CF and CFTR-RD patients globally.

Also, CUAVD has been given less attention in clinical practice. Recent meta-analysis demonstrated 5T and F508del as the most common CFTR abnormalities in CUAVD men [43]. Additionally, if there is a delay in the diagnosis of CUAVD, it may lead to increased mortality and morbidity due to urogenital defects [56]. Taking into consideration the high frequency of renal anomaly risks in men having CUAVD, it is essential to conduct imaging of urogenital system to improve the quality of life and also provide whole exon/flanking sequencing of CFTR to avoid genetic risks to progeny [43].

Review Criteria

A thorough search of medical literature was done on genetics of vas aplasia using the following search engines: PubMed, Google Scholar, Medline, and Science Direct. The keywords "vas aplasia," "genetics," "cystic fibrosis," "azoospermia," "CFTR," "CBAVD," "CUAVD," "CFTR and renal anomalies," and "genetic counseling" were used for the study identification and data extraction.

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Chapter 14
Anomalies of the Y Chromosome

Matheus Roque and Igor Faria Dutra

Key Points

- Although rare in the general population, Y-chromosome microdeletions may be present in up to 7% of severe oligozoospermic and in approximately 15% of azoospermic men.
- In general, patients presenting with a Y-chromosome microdeletion are asymptomatic, although they may present with a reduction in testicular volume.
- Men presenting with <5 million/mL sperm or azoospermia upon sperm analysis must be investigated for a Y-chromosome microdeletion.
- Y-chromosome microdeletion cannot be detected by standard karyotyping, and its diagnosis consists of a series of PCR amplifications.
- Sperm retrieval procedures should be avoided in AZFa patients, and there is controversy as to whether it should be performed in AZFb and AZFbc patients; further, sperm can be found in around 50–70% of patients with AZFc.
- Performing ICSI procedures with sperm from patients presenting with Y-chromosome microdeletion does not increase the risk of complications to the offspring.

M. Roque (✉)
Department of Reproductive Medicine, Mater Prime – Reproductive Medicine, São Paulo, Brazil

I. F. Dutra
Department of Reproductive Medicine, ORIGEN-Center for Reproductive Medicine, Rio de Janiero, Brazil

Department of Surgery, Division of Urology, Antonio Pedro University Hospital, Fluminense Federal University, Rio de Janiero, Brazil

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Case Scenario

Case 1

A couple with 3-year primary infertility was referred to your office for an evaluation. She is a 30-year-old woman with regular menstrual cycles and patent fallopian tubes and presents with an adequate ovarian reserve (antral follicle count = 17; anti-Mullerian hormone = 3.5 ng/mL). He was diagnosed with nonobstructive azoospermia (NOA) after three seminal analyses were performed. All seminal analyses presented volumes ranging from 2.1 mL to 3.5 mL and pH levels from 7.6 to 8.0. No sperm were found even after centrifugation. The karyotyping serum total testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels were all normal. He presented with a left-side Grade III and right-side Grade II varicocele. Six months ago, the couple performed one in vitro fertilization (IVF) cycle in a reference center in your city, with a micro-testicular sperm extraction (micro-TESE) procedure on the day before oocyte retrieval. No sperm was found, even after a bilateral testicular procedure and an overnight testicular sample culture. She performed the oocyte retrieval and had 15 mature oocytes cryopreserved by a vitrification technique. The couple did not want to use a sperm bank. Their doctor suggested that they wait at least 6 months to perform a repeat biopsy and that they should perform a bilateral microscopic varicocelectomy in the meantime to improve the chances of a successful sperm retrieval.

They arrived at your office seeking your evaluation and advice.

Among the following, what would you suggest?

1. To perform the varicocelectomy, as it has been shown that the sperm retrieval rate in NOA is higher after varicocele repair in patients with clinical varicocele
2. To perform a second micro-TESE with another specialist, without prior correction of the varicocele, as there are no clinical data showing any benefit in this strategy before testicular biopsy
3. To use antioxidants, clomiphene citrate, and human choriogonadotropin (hCG) for at least 2–3 months, followed by a reevaluation of the sperm sample
4. To complete the genetic evaluation of the male partner, as it was not complete, although it does not have a prognostic effect on the client's clinical management
5. To complete the genetic evaluation of the male partner, as it was not complete, and it has prognostic effect on the client's clinical management

You suggested that they complete the genetic analysis and perform a test to evaluate possible Y-chromosome alterations. After completing the test, he came to your office presenting with AZFa microdeletion. What do you suggest now?

1. To perform the varicocelectomy, as it has been shown that the sperm retrieval rate in NOA is higher following varicocele repair in patients with clinical varicocele.
2. To perform another micro-TESE with another specialist, without prior correction of the varicocele, as there are no clinical data demonstrating any benefits associated with this strategy before performing a testicular biopsy.

3. To use antioxidants, clomiphene citrate, and hCG for at least 2–3 months, followed by performing another micro-TESE.
4. To look for a sperm sample in a sperm bank to perform intrauterine insemination (IUI).
5. To look for a sperm sample in a sperm bank to perform IVF with her frozen eggs.
6. To suggest options 4 and 5, as they are both correct.
7. Adoption as sperm bank is not always allowed due to religious and cultural or even personal reasons.

Introduction

It has been estimated that 8–15% of couples are unable to conceive after 1 year of regular and unprotected sexual intercourse, at which point they are considered infertile. The male factor is present solely in around 20% of these couples and is associated with a female factor of around 30–40% [1]. The concerted actions of diverse hormones, local secretory factors, and testis-specific genes are necessary for an adequate spermatogenic process. Any defect in this process can lead to the accumulation of errors, resulting in impaired spermatogenesis and, consequently, to male infertility [2]. Extraordinary advances have been achieved in the field of male infertility in the last decades, mainly based on understanding the genetic functions related to testicle development and spermatogenesis [3]. Genetic abnormalities may be associated with problems in the production and transport of sperm and may be present in the form of chromosomal, genetic, nucleotide, or epigenetic modifications and may represent one of the most clinically important aspects of male factor infertility [4].

When compared to fertile men, patients with nonobstructive azoospermia or severe oligozoospermia (<5 million/mL) have a higher risk of presenting with a genetic abnormality [5]. However, even when considering that many azoospermic and oligozoospermic patients present with a genetic predisposition to infertility, the cause (in most cases) remains unknown [6]. Among the known genetic causes of male infertility, numeric and structural chromosomal abnormalities, Y-chromosome microdeletions, X-linked, and autosomal gene mutations have been described [7, 8]. In this chapter, we focused on the genetic causes of male factor infertility related to Y-chromosome alterations.

Background

The first explanation of the role of the Y chromosome in spermatogenesis was proposed by Tiepolo and Zuffardi in 1976, when they identified microdeletions on the long arm of the Y chromosome in six azoospermic patients. They proposed that important genes related to spermatogenesis should be present in this region, which they called the “azoospermia factor” (AZF) region [9]. Thereafter, Vogt et al. (1996)

identified three regions in which the microdeletions were present and that were correlated with the testicular histology related to azoospermia. Thus, they divided the AZF regions into three subregions: AZFa, AZFb, and AZFc. Deletions in this region were identified among 13 out of 370 men presenting with severe oligospermia or azoospermia [10].

Prevalence of Y-Chromosome Anomalies

It has been estimated that Y-chromosome microdeletions are presented in anywhere from 1 in 2000 to 1 in 3000 men. However, this incidence increases to rates of around 7% among infertile men with severely impaired spermatogenesis and to 16% among azoospermic patients [11], although marked differences are reported in different areas around the world [12].

The Genetic Basis for Investigating Y-Chromosome Anomalies

The Y chromosome primarily harbors gene-encoding factors, which are essential for testicle development and are also involved in the process of spermatogenesis. Historically, the Y chromosome has differentiated upon acquisition of the testis-determining gene, followed by large-scale inversions and sequential suppression of X- and Y-chromosome recombination in a stepwise fashion [13–15]. As a consequence of evolution, and to promote function specialization that is selectively advantageous for males [15, 16], progressive genetic decay has occurred, reducing the number of genes on the Y chromosome. This number is much lower than the number of genes on the X chromosome. In the Y chromosome, there are 54 protein-coding genes versus around 700 in the X chromosome [2].

The human Y chromosome is an acrocentric chromosome composed of two pseudoautosomal regions (PAR1 and PAR2) containing 27 genes that encode for products related to diverse biological functions, as well as the male-specific Y region (MSY) that accounts for approximately 95% of the Y chromosome's length. The PARs contain at least 29 genes, presenting diverse roles in cell signaling transcription regulation and mitochondrial function. However, wide differences concerning genetic content and function occur between the two PARs [17]. Defects in the PAR1 genes are associated with mental and stature disorders [18, 19], schizophrenia, and bipolar disorder [20, 21]. Thus, PAR is not related to fertility problems [22]. The MSY is an euchromatic region that encompasses the pericentromeric region and the short (Yp) and long arms (Yq) of the Y chromosome; it has been suggested that MSY plays important health-related roles, involved in processes such as sex determination and brain function regulation [2] (Fig. 14.1).

There is a specific region on the long arm of the Y chromosome (Yq11) that contains 26 genes related to the process of spermatogenesis, the so-called AZF

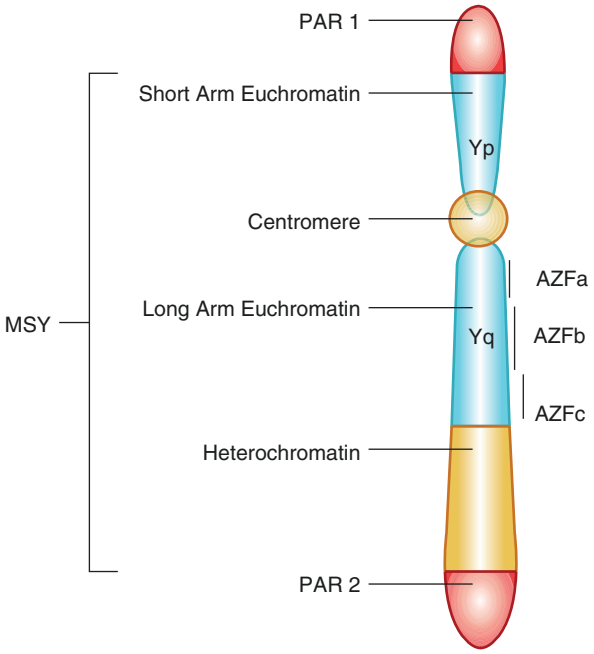

Fig. 14.1 Y-chromosome structure

regions [12, 23], that were first described by Vogt et al. (1996) [10]. These genes are organized into three distinct locations: the so-called a (AZFa), b (AZFb), and c (AZFc) regions. Deletions in these regions, which may occur independently or in association, may lead to severe oligozoospermia or even azoospermia [2, 7]. The effect on spermatogenesis depends on the AZF subregions affected. The most frequent microdeletion subtype is the AZFc region, which accounts for 80% of AZF microdeletions. Further, AZFb occurs in 15% of Y microdeletions, while AZFa is rare and accounts for less than 3% of them [23, 24].

The MSY genes related to spermatogenesis are classified into two categories: single-copy and ampliconic multicopy genes. In the **AZFa** region, there are two protein-coding genes related to spermatogenesis: *USP9Y* and *DBY. DBY* plays a major role in spermatogenesis, encoding RNA helicase. Partial AZFa deletions, with USP9Y deleted in isolation, have been reported. The **AZFb** region contains seven protein-encoding genes related to spermatogenesis, including *EIF1AY*, *RPS4Y2*, and *SMCY*, which are located in the X-degenerate euchromatin, and *HSFY*, *XKRY*, *PRY*, and *RBMY*, located in the ampliconic regions. The **AZFc** region contains five protein-encoding genes related to spermatogenesis: *BPY2*, *CDY*, *DAZ*, *CSPG4LY*, and *GOLGAZLY*. The *DAZ* (a deleted gene in azoospermia) was the first candidate gene to be isolated from the AZFc region, was identified as a frequently deleted gene on the Y chromosome of infertile males, and has been the most studied [7]. It was later found that *DAZ* is subdivided into four genes (*DAZ 1* and *2* and *DAZ 3* and *4*) and that these four copies are expressed in spermatogonia and in all stages

of germ cell development [2]. There are also genes outside the AZF regions that have been thought to play a role in gametogenesis; however, neither the deletion nor the mutation of these genes has been identified in infertile patients, and there is still a lack of evidence of their spermatogenic function [25].

A large number of genes of the Yq locus are transcribed in the testis and play a fundamental role in spermatogenesis; as such, the loss of these regions would be tied to infertility. In this way, Y-chromosome infertility may be caused by microdeletions or rearrangements of the Yq arm of the Y chromosome in the AZF regions that are associated with the deletion, duplication, and variations of multiple genes. Clinically, Y-chromosome alterations can be classified as (1) AZF deletions, occurring with the complete loss of one or more AZF loci; (2) partial AZF deletions and duplications; and (3) gene copy number variations (CNVs). These modifications are related to different testicular histology and to a patient's prognosis [2].

In clinical practice, the AZFa, AZFb, and AZFc deletions are among the leading causes of spermatogenic failure, indicating that the need to screen for AZF deletion should be part of the routine diagnostic workup for infertile man [8, 23, 26].

Clinical Presentation and Diagnosis of Men with Y-Chromosome Anomalies

Males with Y-chromosome infertility are generally asymptomatic, although they may present a reduction in testicular volume. Thus, it is of utmost importance to adequately evaluate men with sperm counts <5 million/mL or those with azoospermia, as they present with a higher risk of genetic abnormalities; thus, it may be necessary to perform genetic counseling [1, 8]. However, in some cases in which the Yq deletions extend close to the centromere in a region that contains a putative growth-controlling gene (*GCY*), short stature may occur [27, 28]. This can also be caused by hidden copy number changes within PAR [29].

Y-chromosome microdeletions cannot be detected by standard karyotyping [8], and their diagnosis consists of a series of polymerase chain reaction (PCR) amplifications within relatively broad regions of the Y chromosome [7, 8]. A series of PCR amplifications within the broad regions of the Y chromosome make it possible to molecularly diagnose Y-chromosome microdeletions. There are many specific genes (*USP9Y*, *DDX3Y*, *BPY/VCY*, *HSFY1*, *HSFY2*, *KDM5D*, *RPS4Y2*, *RBMY*, *PRY*, *DAZ*, and *CDY*) located in the long arm of the Y chromosome that should be included in the analysis of Y-chromosome integrity [30].

Genetic Counseling

Spermatozoa from infertile patients with Yq microdeletions are fully fertile; they retain their fertilization potential after intracytoplasmic sperm injection (ICSI) and even for natural conception [31]. Although a severe impact on spermatogenesis can

be observed in these patients, there are some accounts of infertile men that have spontaneously fathered a child in cases of severe oligozoospermia without any associated female factor infertility. However, this is rare, and – in general – the patients will need to perform IVF using ICSI techniques. Y-chromosome infertility is inherited in a Y-linked manner, and the male offspring will have the same deletion from their father and will present with risks of facing infertility problems in the future [3]. However, the severity of spermatogenesis failure cannot be entirely predicted given different genetic backgrounds and the presence or absence of environmental factors that may jeopardize the patient's reproductive function [31]. There are no consequences for female offspring without the risk of congenital anomalies [3].

Management and Prognosis

Infertile men presenting with Y-chromosome anomalies and exhibiting severe oligozoospermia or azoospermia may father their child with the use of ICSI when sperm is available in the ejaculate or can be found in a testicular sperm retrieval procedure [32, 33]. Different testicular phenotypes are presented depending on the type of deletion, and these differences are related to the chance of obtaining sperm during a testicular procedure. However, the presence of deletion has no impact on fertilization and pregnancy during IVF/ICSI treatment and is not associated with an increased risk for birth defects [34]. Molecular diagnosis and subtyping the microdeletion are useful not only for genetic counseling but also for counseling the patient about his chance of presenting sperm during a testicular sperm extraction procedure [24, 34–36].

There are six classic forms of AZF microdeletions, and they are related to the phenotype. These forms are (1) AZFabc, related to the Sertoli-cell only (SCO) histopathology, (2) AZFa (SCO), (3) AZFbc (SCO/maturation arrest), (4) AZFb (maturation arrest), (5) AZFc (severe oligospermia to azoospermia), and (6) partial AZFc (normal spermatogenesis to azoospermia) [37]. Patients presenting with the AZFa microdeletion are invariably associated with pure SCO histopathology in their testicular samples and should be advised to avoid undergoing a sperm extraction procedure, as no sperm are found in this type of microdeletion [34, 36, 38].

Thus, identifying the Y-chromosome microdeletion holds diagnostic, prognostic, and preventive value. In this way, the diagnosis of a Y-chromosome microdeletion can explain the reason underlying a patient's infertility, thereby avoiding unnecessary medical and surgical treatments. Such treatments can involve performing unnecessary procedures with the main goal of improving the seminal characteristics, if the reason underlying the infertility can be identified as a genetic cause; these treatments are not reversible [35].

Depending on the pattern of the Yq microdeletion, patients presenting with azoospermia may be subject to testicular sperm retrieval. The chance of retrieving sperm in an AZFa microdeletion is virtually null, and sperm retrieval should be avoided [12, 24, 34, 36, 38, 39]. Still controversial is whether patients presenting with AZFb and AZFbc should undergo sperm retrieval, as their prognosis is very

poor. However, there are some cases of AZFb microdeletion that present with oligozoospermia, suggesting that sperm may be found in azoospermic patients with this microdeletion [36, 40, 41]. In contrast, patients with AZFc microdeletions usually have residual spermatogenesis, and the chance of success of a sperm retrieval procedure is around 50–70%. Moreover, the chances of fatherhood via ICSI when sperm are found in patients with an AZFc microdeletion seem to be unaltered [24].

Conclusion

Among the known genetic causes of male infertility, Y-chromosome microdeletion is an important cause of severe oligozoospermia and azoospermia and should thus be evaluated in these cases. Its investigation not only has a diagnostic purpose but also holds prognostic and preventive value. Patients should be counseled on the fact that when sperm is present, performing ICSI will not increase the risk for complications in their offspring, and these patients may genetically father their children.

Review Criteria
A thorough search of medical literature was conducted with the MEDLINE, EMBASE, ScienceDirect and Scielo databases until November 2018. We used relevant terms, namely, "Y chromosome anomalies," "Y chromosome microdeletion," "genetic cause," and "male infertility."

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Chapter 15
Infertility and Cryptorchidism

Joshua Bitran, Simon Dadoun, and Ranjith Ramasamy

Key Points

- Cryptorchidism is the most frequent congenital birth defect in male children and represents an important risk factor for infertility and testicular cancer.
- Almost one in ten infertile men has a history of cryptorchidism.
- Low birth weight and gestational age have consistently shown a strong association with cryptorchidism.
- The testes require a lower temperature than the abdomen in order for spermatogenesis to occur—cryptorchidism disrupts this process and therefore impairs fertility.
- It is recommended that orchiopexy be performed as early as 6–12 months of age to ensure that optimal germ cell development occurs during the first year of life.

Case: A 26-year-old male married to a 24-year-old female complains of inability to conceive after 2 years of unprotected intercourse. At the age of 7, he had a unilateral orchiopexy for undescended testis. Physical examination demonstrates bilateral 8 cc firm testes. Hormonal evaluation demonstrates FSH 12 IU/mL, LH 8 IU/mL, and T 223 ng/dL. Semen analysis demonstrates normal volume, normal pH, and azoospermia. What is the best management of this patient?

J. Bitran · S. Dadoun · R. Ramasamy (✉)
Department of Urology, University of Miami, Miami, FL, USA
e-mail: ramasamy@miami.edu

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Introduction

Cryptorchidism is a condition in which one or both of the testes become arrested somewhere along their path of migration from the posterior abdominal wall to the scrotum. It is one of the most frequent congenital birth defects in male children and represents an important risk factor for infertility and testicular cancer. Cryptorchidism should not be confused with testicular ectopia, a condition in which the testes take on an abnormal location outside the migration tract. Cryptorchidism can occur unilaterally or bilaterally (20–40% of all cryptorchidism cases) [1].

For practical purposes, cryptorchidism can be organized into distinct clinical categories, such as congenital or acquired, palpable or non-palpable, and unilateral or bilateral. This chapter deals with congenital cryptorchidism, as the acquired form occurs as the result of an inguinal surgery such as orchiopexy or inguinal hernia repair. A palpable undescended testicle can be felt in the inguinal canal. If the testis is non-palpable, it means we are dealing with an abdominal testis or a complete lack of that testis, termed *anorchia.*

Prevalence

Cryptorchidism is the most frequent congenital birth defect in male children and represents an important risk factor for infertility and testicular cancer. Approximately 2–4% of full-term newborns are born with at least one cryptorchid testis. In preterm births, the rate increases to about 30%. During the first few months of life, cryptorchid testes may continue their normal descent, so the prevalence decreases to 1% by the end of the first year [2].

Risk Factors

Cryptorchidism is still largely considered to be idiopathic in nature. However, emerging research points to various genetic and environmental factors thought to disrupt the hormonal pathways that regulate testicular descent.

Evidence supporting genetic causes of cryptorchidism is abundant. Familial cases have been described, and a family history for cryptorchidism represents a risk factor for undescended testes [3]; initial studies suggested that the brothers and fathers of patients with cryptorchidism had a higher chance of having cryptorchidism as well [4]. More recent data highlighted the fact that 22.7% of patients with undescended testis have a positive family history vs. 7.5% in controls [5]. More than 250 entries for cryptorchidism are present in OMIM, the Online database of Mendelian Inheritance in Man, which catalogs all human genes and genetic disorders. This result points out the complexity of the pathogenesis of cryptorchidism, whether existing as an isolated symptom or associated with other anomalies [6].

Compared to children conceived naturally, children conceived with the aid of assisted reproductive technologies (ART) are 30% more likely to have a congenital malformation such as cryptorchidism [7]. The causal mechanism is largely thought to be the propensity of ART babies to be born preterm or with low birth weight. Research also suggests that parental subfertility itself is a risk factor for congenital malformation. Children of parents who took longer than 12 months to conceive naturally are 20–40% more likely to have some congenital malformation compared to those who took less than 12 months to conceive [8].

Over the years, numerous drugs used during pregnancy have been posited for an association with cryptorchidism. Among those implicated are DES (diethylstilbestrol) [9], prenoxdiazine (cough medication) [10], and, most notably, analgesics such as acetaminophen and ibuprofen [11, 12]. While some of these associations have garnered significant media attention—especially drugs as ubiquitous as analgesics—most of these studies had relatively small sample sizes, and thus their power to detect statistically significant associations was limited. In the case of analgesics, the conflicting evidence makes it especially difficult to justify an association between maternal use of analgesics and cryptorchidism. For every study suggesting a strong positive association, there is another limited [13, 14] or zero [15] evidence. Because of this conflicting evidence, it has been suggested that rather than having a direct causal relationship with cryptorchidism, increased maternal intake of analgesics may be an indicator of other, yet unmeasured factors taking place [16]. The ubiquity of analgesics in the western pharmacy cabinets points to the need of a well-powered, multivariate-adjusted investigations to better assess this exposure.

Finally, one cannot overlook the various environmental or occupational exposures that have been associated with cryptorchidism. Multiple studies have investigated the association between prenatal environmental exposure of these chemicals and the development of cryptorchidism. For the most part, the chemical exposures of interest are synthetically manufactured chemicals—particularly pesticides used in agriculture. In one study, researchers observed in a large Danish cohort that sons of mothers who farmed during pregnancy were nearly one third more likely to develop cryptorchidism compared to sons of mothers who did not farm [17]. Another cohort study observed that sons born to women who lived in areas sprayed with dichlorodiphenyltrichloroethane (DDT) were more than twice as likely to be born with cryptorchidism [18]. Studies performed in Hungary [19] and South Korea [20] observed that the risk of cryptorchidism increased with proximity to chemical plants. While these observations provide little insight as to which specific exposures are associated with cryptorchidism, they do suggest that exposure to potential endocrine-disrupting chemicals as a whole may increase the risk of cryptorchidism.

It is worth noting that low birth weight and gestational age have consistently shown a strong association with cryptorchidism [21–23]; however, rather than being causal risk factors per se, birth weight and fetal growth restriction may have a shared etiology with cryptorchidism. The inguinoscrotal phase of testicular migration occurs in the last trimester of pregnancy, and therefore, factors which result in premature birth or that limit completion of fetal development tend to be associated with disorders of testicular descent.

Testicular Descent

Testicular descent is thought to occur in two stages, with each phase being governed by a combination of hormonal influences and growth processes [24]. The transabdominal phase is characterized by the descent of the testis into the lower abdominal position, while the latter, inguinoscrotal phase, describes the passage of the testis through the inguinal canal and into the scrotum.

The first stage of testicular descent occurs between 10 and 15 weeks of gestation. During this phase, the testes remain close to the future inguinal region during enlargement of the abdominal cavity and under the pressure of the abdominal visceral growth. The gubernaculum testis also plays a decisive role in this phase of testicular migration. Rostrally, it has its origin at the caudal end of the testis and inserts in the region of the genital swelling (future scrotum). Simultaneously, at the insertion point of the lower gubernaculum, an invagination of the peritoneum arises (the "processus vaginalis"). The peritoneal invagination deepens while the gubernaculum shortens, thereby pulling the testis downward. Indeed, the name "gubernaculum," Latin for rudder, aptly describes this organ's role in steering the testis into the scrotum.

INSL3 is a small peptide hormone expressed in testicular Leydig cells and is first detected right before the onset of testicular descent. INSL3's receptor, the *relaxin family peptide 2 (RXFP2)*, is expressed on the gubernaculum. The presence of both INSL3 and its receptor is necessary for the swelling and maturation of the gubernaculum [25]. Anti-Mullerian hormone (AMH) has also been suggested to have a role in the development of the gubernaculum. Boys with AMH mutations are born with persisting Mullerian ducts and intra-abdominal undescended testes but normal masculinization of the external genitalia. Combined with the fact that an androgen deficiency does not affect this first phase of transabdominal descent, one can assume that INSL3 and AMH are the primary hormones responsible for the first phase of the testis' migration.

Unlike the transabdominal phase, the inguinoscrotal phase of testicular descent occurs in the last trimester of pregnancy and is dependent on testosterone. During this phase, the testes move from the inguinal region to the scrotum. Testosterone acts on the cranial suspensory ligament (CSL), causing it to regress. Testosterone also acts on the gubernaculum. The androgens may masculinize the sensory branches of the genitofemoral nerve, which then releases a neurotransmitter, calcitonin gene-related peptide (CGRP), to control the growth and elongation of the gubernaculum. The diameter of the gubernaculum reaches its maximum during the seventh month, which induces widening of the surrounding inguinal canal. Simultaneously, the tip of the processus vaginalis actively elongates to create a peritoneal diverticulum which will allow the intra-abdominal testis to leave the abdomen [6] (Fig. 15.1).

Interestingly, a spike in androgen production in human embryos directly precedes inguinoscrotal testicular descent [26]. Suppression of androgen production

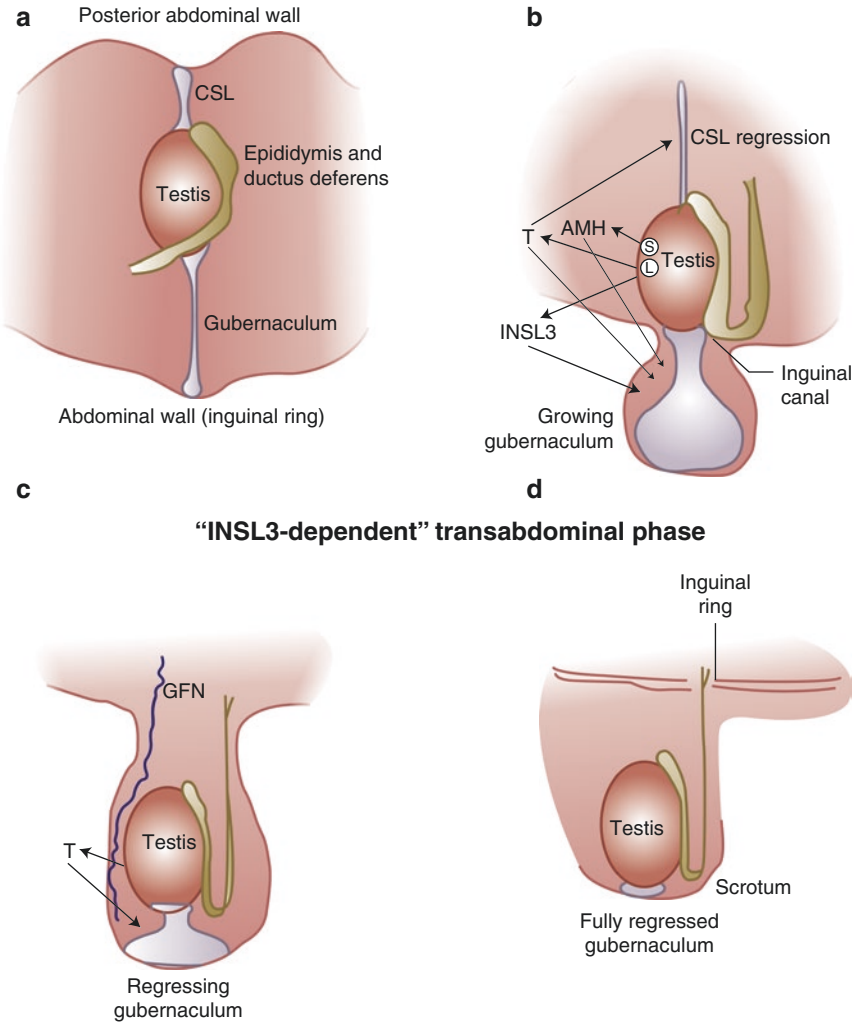

Fig. 15.1 Model of testicular descent in humans, showing the INSL3-dependent transabdominal phase (**a**, **b**) and the androgen-dependent inguinoscrotal phase (**c**, **d**). The major structures and the roles of hormones are shown. Testicular differentiation from the ambisexual gonad in the presence of the Y chromosome (**a**) led to the production of AMH from the developing Sertoli cells (S) and production of testosterone (T) and INSL3 from the Leydig cells (L) (**b**). The direct and indirect (via the GFN and CGRP) effects of these two hormones principally on the CSL and gubernaculum cause the two-step process of testicular descent. Regression of CSL is mainly under the control of testosterone (**b**). Masculinization of gubernaculum is under the major control of INSL3 (**b**), whereas minor roles seem to be exerted by AMH and androgens, possibly via the GFN and CGRP [6]

and/or androgen receptor deficiency/inhibition has been linked to cryptorchidism in humans and various other species [27]. Because androgens also govern the differentiation of external sexual organs in males, it is not surprising that male neonates who present with genital malformations such as hypospadias (a result of poor androgen signaling) may also present with varying degrees of cryptorchidism.

Cryptorchidism can manifest itself as a variety of presentations. Exactly where the affected testicle's migration has been arrested can shed light on its etiology and pathogenesis. For example, an abdominal testicle may indicate a mutation in the AMH, INSL3, or RXFP2 genes, while a testicle found in the inguinal canal may indicate intrauterine exposure to estrogen or an androgen receptor gene mutation.

Cryptorchidism and Infertility

In normal development, the testes descend from the abdomen to the scrotum to encounter the lower ambient temperature necessary for spermatogenesis to occur (Fig. 15.2). The unfavorable environment surrounding the undescended testes impairs both differentiation of the fetal gonocytes (fetal/neonatal primordial germ cells) into spermatogonia and the programmed germ cell death of the remaining

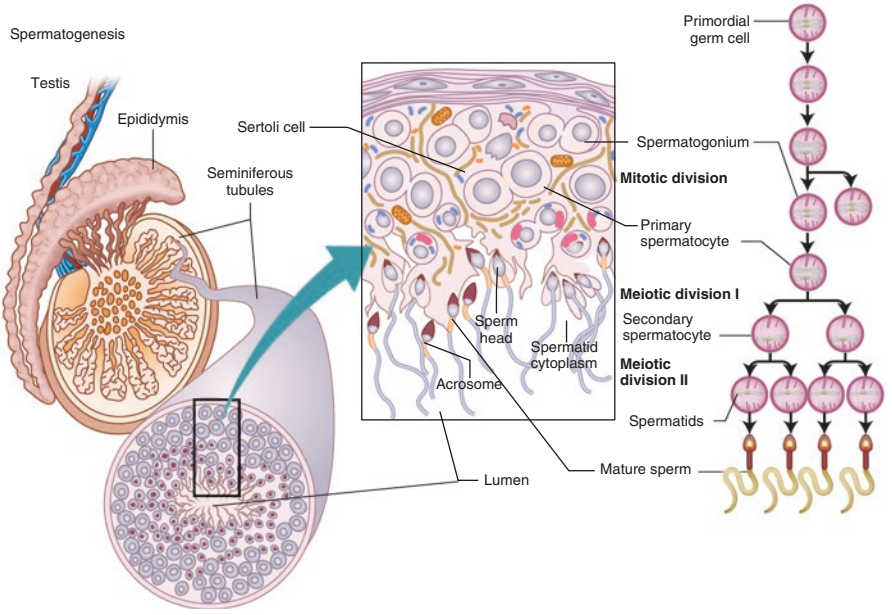

Fig. 15.2 Spermatogenesis. Beginning at puberty, dividing spermatogonia along the basement membrane of the seminiferous tubules provide a constant turnover of primary spermatocytes and spermatogonia through mitosis. Primary spermatocytes eventually become mature sperm. Excess heat due to cryptorchid testes damages the primordial germ cells (gonocytes) that become spermatogonia and therefore can cause infertility [45]

undifferentiated gonocytes. The lack of spermatogonia means that there are less stem cells for postpubertal spermatogenesis, while the persisting undifferentiated fetal gonocytes can become malignant after puberty [28]. Indeed, the goals of bringing cryptorchid testes down to the more favorable environment of the scrotum is to preserve the potential for spermatogenesis and also to allow for the differentiation of fetal gonocytes into spermatogonia and therefore decreasing the amount of undifferentiated fetal gonocytes that can potentially become malignant.

Almost one in ten infertile men has a history of cryptorchidism. Long-term outcome studies have shown that a history of cryptorchidism in the past was associated with a 30–60% risk of infertility or lack of germ cells in adult men. Men with a history of bilateral cryptorchidism have decreased fertility compared to their unilateral counterparts. 89% of adult men with untreated bilateral cryptorchidism are diagnosed with azoospermia (a complete lack of sperm in the ejaculate). It is interesting to note that while men with a history of unilateral cryptorchidism have a lower fertility rate, they have a paternity rate similar to the normal population. On the other hand, adults with a history of bilateral UDT have lower fertility and paternity rates [1].

Cryptorchidism and Cancer

Lack of germ cells has been reported as early as 12 months of age, and therefore orchiopexy has been recommended before 12 months of age. Carcinoma in situ is more common in men with a history of cryptorchidism, with the prevalence of 2–3% in adult patients [29]. The incidence of CIS is higher in men with macroscopic testicular atrophy, bilateral cryptorchidism, intra-abdominal testes, abnormal genitalia, or an abnormal karyotype [30]. In boys with unilateral UDT (undescended testis), the contralateral descended testis has a slightly increased risk of cancer [31].

There is clinical evidence from long-term follow-up studies that cryptorchidism in the past is associated with a 5–10 times increase in TGCT (testicular germ cell tumors) [32]. Testicular germ cell tumors are common, affecting 1% of young men [29]. They have a prevalence of 1% of all neoplasms in men, with the peak incidence between 20 and 30 years of life. About 10% of all cases of TGCT develop in men with a history of cryptorchidism. The risk of TGCT is greater in intra-abdominal and bilateral UDT [32, 33]. The risk of TGCT is significantly decreased if orchiopexy for UDT is performed before puberty [34]. Now, it is recommended that orchiopexy be performed as early as 6–12 months of age to ensure that optimal germ cell development occurs during the first year of life [35, 36].

Treatment

The main objective in the treatment of cryptorchidism is to facilitate the descent of the testis down into the scrotum. This is done primarily to prevent the impairment of spermatogenesis and to minimize the risk of TGCT. The two approaches

are hormonal and surgical which can be used alone or, more commonly, in combination.

The *hormonal* treatment of UDT is based on the hypothesis that a lack of androgens in utero is responsible for the arrest of the testis along its migratory path. As androgens take part in the process of testicular descent, it seems justified to use hormone therapy to stimulate its endogenous production. The hormones most commonly used are hCG, gonadotropin-releasing hormone (GnRH), or a combination of both. Hormonal therapy can be administered as a neoadjuvant therapy prior to the orchiopexy or as a supplementary treatment after early surgery for UDT [28]. The administration of hCG goes back as early as the 1930s [37]. In utero, hCG is produced by the syncytiotrophoblasts of the implanted embryo and stimulates testicular Leydig cells to produce testosterone. Treatment with hCG is still used to this day. However, the use of hCG in treatment has given way to GnRH following critical studies and meta-analyses in the 1990s and 2000s indicating that hCG is associated with adverse effects on future reproductive function [38, 39].

GnRH is produced by the hypothalamus and stimulates the anterior pituitary gland to secrete LH and follicle-stimulating hormone (FSH). FSH stimulates the proliferation and differentiation of spermatogonia. GnRH therapy may improve germ cell number, maturation, and later semen parameters in boys with cryptorchidism [34, 35]. The combined administration of GnRH and hCG in boys younger than 1 year can be beneficial for spermatogonial transformation and proliferation with a success rate of about 20% [40].

Nowadays, the surgical therapy for the *palpable* UDT is orchiopexy. Fixation is achieved by the scarring of the tunica vaginalis to the scrotum. If the testis is non-palpable, diagnostic laparoscopy is the procedure of choice. *Non-palpable* testes create a few clinical situations. Testes can be present in the intra-abdominal position, or one or both testes can be missing (anorchia). Positive testosterone response to hCG stimulation, low serum levels of FSH, and normal levels of inhibin B confirm the presence of functioning testicular tissue. No response to hCG stimulation, increased serum levels of FSH, and very low levels of inhibin B in boys with bilateral non-palpable testes prove that testicular tissue is not present [41]. Currently, orchiopexy is recommended between 6 and 12 months of age [42, 43]. However, the risk of poor sperm count is probably independent of the age of surgery, but it is correlated with the number of gonocytes and spermatogonia present [35]. It is important to note that in addition to promoting spermatogenesis and minimizing the risk of TGCT, surgery also helps to minimize the risk of torsion of the testis, which is increased in cryptorchid infants due to the greater mobility of the inguinal testis and patent processus vaginalis [44].

Normal testicular descent is a process largely governed by a carefully orchestrated hormonal symphony. The transabdominal phase is characterized by the descent of the testes into the lower abdominal position, which is primarily governed by INSL3. The inguinoscrotal phase describes the passage of the testes through the inguinal canal and into the scrotum and is largely governed by androgens. By understanding the normal physiology of testicular descent, the location of the undescended testes can shed light on which processes was interrupted.

There are a variety of genetic and environmental risk factors that can impact the precise timing and coordination of these processes, resulting in cryptorchidism. Understanding the pathophysiology is important because the main complications associated with cryptorchidism are infertility and cancer. Timely treatment can minimize the risk of developing these complications. Current treatment focuses on hormonal and surgical methods in order to direct the testes in the favorable environment of the scrotum.

Review Criteria
A thorough search of medical literature was done on cryptorchidism using the following search engines: PubMed, SCOPUS, and ScienceDirect. The keywords "cryptorchidism," "infertility," "testicular descent," "mechanism of descent," "cryptorchidism management," and "spermatogenesis" were used for study identification and data extraction.

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Chapter 16
Kartagener and Immotile Cilia Syndrome

Igor Faria Dutra and Matheus Roque

Key Points

- Although Kartagener syndrome (KS) is a heterogeneous group of disorders with similar clinical presentations, the associated infertility treatments have undergone much evolution.
- It is worth emphasizing that medical conduct should always be individualized, with sperm motility serving as an extremely relevant factor in decision-making.
- ICSI represents a successful milestone for coping with infertility in men with KS, in which spermatozoa are either completely evident or initially immobile in the ejaculate.
- The use of testicular spermatozoa in combination with ICSI may serve as an alternative treatment and may be associated with even better results when compared to ejaculate sperm.
- Genetic counseling is strongly recommended for these patients.

I. F. Dutra
Department of Reproductive Medicine, ORIGEN-Center for Reproductive Medicine, Rio de Janiero, Brazil

Department of Surgery, Division of Urology, Antonio Pedro University Hospital, Fluminense Federal University, Rio de Janiero, Brazil

M. Roque (✉)
Department of Reproductive Medicine, Mater Prime – Reproductive Medicine, São Paulo, Brazil

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Case Scenario

A couple presenting with 2.5 years of primary infertility was referred to your office. They have performed an initial infertility workup with the gynecologist and a previously failed in vitro fertilization (IVF) treatment at another center. She is 25 years old and presents with no infertility factor. He is 32 years old and presents with a history of recurrent respiratory disease. His parents were consanguineous, and his brother is also facing infertility problems. The patient denied the use of illicit drugs. Two seminal analyses were performed and presented with a normal volume, pH level, and sperm concentration. However, in both seminal analyses, total asthenozoospermia was evident. There were no alterations on genital examination, and he presented with a normal testicular volume, epididymis, vas deferens, and no varicocele. The couple has performed one IVF treatment at another center, presented with a good ovarian response to the controlled ovarian stimulation with the retrieval of 18 oocytes, of which 15 were metaphase II (mature oocytes). Due to total sperm immotility, intracytoplasmic sperm injection (ICSI) was performed with ejaculate sperm; however, none of the injected oocytes were fertilized.

At your center, you suggested performing another IVF cycle with an ICSI procedure using testicular sperm. Prior to treatment, the couple underwent genetic counseling, after which they performed ICSI treatment. She got pregnant after the first embryo transfer and had a pregnancy without any obstetrical or perinatal complications, delivering a healthy, full-term baby.

Introduction

A diagnosis of infertility is established when a couple is unable to conceive after 1 year of regular and unprotected sexual intercourse. It has been estimated that 8–15% of couples in reproductive age present with infertility, and the male factor is present in around 50–60% of these couples. Male infertility is present solely in around 20% of infertile couples and is associated with a female factor in around 30–40% [1]. Among the many causes of male infertility, Kartagener syndrome (KS) – also known as immotile cilia syndrome or primary ciliary dyskinesia (PCD) – is among its genetic causes and deserves special attention, as it is accompanied by many complications that may severely affect the patient's quality of life [2].

PCD is a genetically heterogeneous autosomal recessive disorder, characterized by an abnormal or inefficient functional pattern of the cilia and flagella of the human body, resulting in incompetent mucociliary clearance and mucus retention [3–5]. This problem results in an array of clinical manifestations, including the classic triad of chronic sinusitis, bronchiectasis, and situs inversus, as well as male infertility due to the immobility of the spermatozoa in the patient's ejaculate [6, 7]. Approximately 50% of patients with PCD have lateral defects, such as dextrocardia or situs inversus totalis, thus characterizing KS [8]. It has a preva-

lence rate of 1 in 15,000 to 20,000 births. However, it has been hypothesized that this number is underestimated, as just a few patients with PCD carry a well-established diagnosis [9].

Concerning fertility, ciliary dysfunction may affect both male and female fertility. As the sperm flagellum is a type of cilia, an abnormal ciliary structure may lead to the reduction or inability of the flagellum to adequately work; as a consequence, male infertility may occur. On the other hand, ciliary dysfunction may also occur in the fallopian tubes of affected women, leading to an increased risk of ectopic pregnancy or infertility [10]. In this chapter, we will perform a review of the literature related to PCD and focus on its relevance in male infertility.

Historical Background

PCD was first reported by Kartagener et al. in 1933, when they described four patients presenting with the triad of chronic sinusitis, bronchiectasis, and situs inversus, thus establishing KS [11]. Forty years later, Afzelius noted that these patients had "immotile" cilia and defective ciliary ultrastructure, specifically noticing a functional change in dynein arms, promoting decreased mucociliary clearance due to a lack of ciliary movement, which is known as "immotile cilia syndrome" [11–13]. Subsequently, when functional ciliary involvement was observed without ultrastructural deformities, as well as moving cilia with abnormal movement patterns that could result in clinical disease, the syndrome was renamed to "primary ciliary dyskinesia" (PCD) [14–16]. This name can more appropriately describe its heterogeneous genetic base and ciliary dysfunction, and it can also distinguish it from secondary ciliary defects acquired after multiple causes of epithelial injury [9]. Nowadays, PCD encompasses all congenital ciliary dysfunctions, and the term KS is reserved when the situs inversus is present in association with the syndrome [10].

Physiopathology

To understand the clinical manifestations of PCD, it is necessary to know the physiological structure of cilia and flagella. Cilia are highly complex and dynamic organelles that protrude on the surface of cells and are supported by a cytoskeleton called axonema, which consists of hundreds of proteins and a complex arrangement of microtubules [17]. Historically, cilia are recognized for their role in cell motility and in the transport of fluids over mucosal surfaces. However, it has been recently proposed that they also have a sensory function that modulates elements of development and cell function. Both motile and sensory cilia are composed of highly organized arrays of microtubules and attendant accessory elements [9].

Structurally, the axonema presents with nine pairs of peripheral microtubules that are formed from α- and β-monomers of tubulin configured into helical patterns

of protofilaments (tubule A and tubule B). They are arranged around two central microtubules, classified as a 9 + 2 configuration. These units are linked by three structures that play an important role in maintaining the overall structure of the flagellum: (1) A nexin link, stabilizing the axonema through an elastic bridge linking consecutive microtubules; (2) dynein arms, formed by the outer dynein arm (ODA) and the inner dynein arm (IDA) and connected by the nexin links. These are responsible for the flagellum swing, as they allow the nine pairs of microtubules to slide against each other; and (3) radiating spokes, which connect the central microtubules with peripheral microtubules and a center sheath. In the absence of the central microtubules, they are characterized as 9 + 0 [9, 18, 19]. Thus, the axonemal dyneins are the molecular motors that generate the sliding of the microtubules through their subunits, which are known as dynein arms, and that which bind to the peripheral microtubules and promote the mobility of cilia and flagella by ATPase-dependent reactions [20] (Fig. 16.1).

Cilia have differences in their structural and axonema motility arrangement, and a variety of structural defects have been found in PCD patients, which may involve a microtubular structure, abnormal radiating spokes, and also the ODA/IDA [21, 22]; for example, the 9 + 0 cilia can be immotile rather than mobile and present as a single cilia on the cell's surface, while the 9 + 2 cilia are mobile and characterized as multiple cilia. Almost all human cells have a nonmotile monocyte, referred to as the primary or sensory cilia, while specialized cells have mobile multis [20, 23, 24].

Thus, structural and functional ciliary problems result in poor or absent ciliary movement, in which the retention of secretions may occur as a consequence of a defective ciliary-clearing function. Moreover, partial or complete situs inversus may

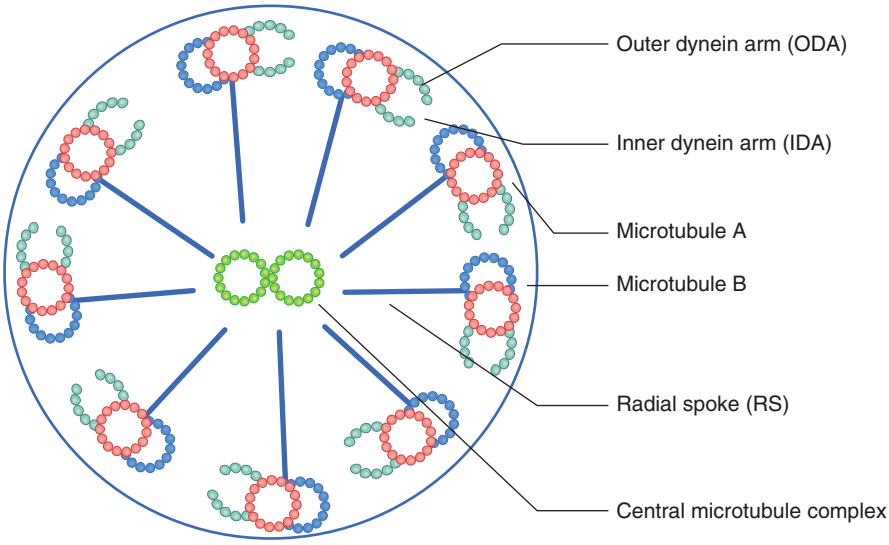

Fig. 16.1 Cross section of major ultrastructural components of normal cilia

result from ciliary disorders, as the cilia are involved in the process of viscera sinus orientation in the embryo by swinging in a certain direction [10].

Genetic Basis for PCD

The PCD has an autosomal recessive or X-linked inheritance. The genetic cause, when known, has been primarily associated with mutations that affect one of the hundreds of genes encoding essential components of the mobile ciliary mechanism complex. Cilia and the axonema contain a variety of structural proteins or regulatory proteins that control hundreds of genes. Modifications in any of these genes may affect the ciliary movement and cause any clinical manifestations. Therefore, PCD is characterized by mutations affecting the axonemal dynein arm motors. Two of the most studied genes in PCD are the axonemal dynein intermediate-chain gene (*DNAI1*) and the axonemal dynein heavy-chain gene (*DNAH5)*, and mutations in these genes may result in the absence of ODA, leading to ciliary ultrastructure dysfunction [25–27]. Mutations can also occur in ODA-targeting and ODA-anchoring proteins, such as CCDC103 and ARMC4, and in complex components (ODA-DC) as CCDC151 [28–34]. Perhaps more surprising than the finding that mutations in the structural components of cilia are a common cause of PCD has been the increasing number of PCD loci that do not encode parts of the cilia itself but rather encode proteins that act in the complex structural process and transport of ciliary dynein [35, 36]. Several additional proteins involved in dynein assembly were identified in nonhuman studies, including ODA8, ODA5, and ODA10 from the flagellate organism, *Chlamydomonas reinhardtii* [37, 38]. To achieve the genetic diagnosis of PCD, autosomal recessive or hemizygous biallelic mutations in an X-linked gene must be identified. Mutations reported in known genes account for only about 70% of cases of PCD; therefore, additional genes have yet to be identified [35].

Clinical Presentation

The PCD is equally incident in males and females, and, in general, most diagnoses are made in school-aged children or adolescents younger than 15 years old [10, 39]. As cilia are widely present in a variety of tissues and organs, clinical PCD may not merely exhibit the typical triad but may often be accompanied by a variety of malformations and complications [10].

The nodal cilia of the left–right embryonic organizer have a 9 + 0 structure, similar to nonmoving cilia (9 + 0), but they have motility. When this ciliary motility is defective, it can lead to changes in the positioning of the body's organs, which has a left–right direction defined in a randomized way. Thus, problems of laterality can occur and can affect approximately half of individuals with the PCD, leading to cardiac pathologies in the spleen and other organs [20, 23, 24, 40].

Both the cilia of the respiratory epithelium, the fallopian tubes, and those which lines the cerebral ventricular system have a 9 + 2 arrangement. Mutations affecting the axonemal dynein arm motors of these cilia promote infection in children and chronic congestion of the upper and lower airways, progressing to bronchiectasis. Symptoms usually appear early in life, with neonatal respiratory distress syndrome, in addition to promoting acute and chronic respiratory infections, such as nasosinusitis and tympanitis, and also leading to bronchiectasis. Affected individuals may also have hydrocephalus, auditory defects, and retinal dystrophy [40–42].

The flagellum and the cilia are quite similar in ultrastructure, although they have subtle differences in the distribution of the dynein arms along the axoneme. When compared to moving cilia, they differ primarily in regard to their length and motility pattern, which undulates like a scarf or a whip on the cilia. The motor in both cases is the same, with the axonema featuring a 9 + 2 arrangement. A functional alteration of axoneal dyneins in the spermatozoa flagella leads to sperm immotility and, consequently, to male infertility [2, 43, 44].

Diagnosis

The "gold standard" diagnostic test for PCD has been the electron microscope ultrastructural analysis of respiratory cilia that can be obtained by a nasal scrape or bronchial brush biopsy. Limited clinical genetic testing is currently available in clinical settings [9]. Nasal nitric oxide (NO) measurement can be used as an adjunct test to identify patients at risk of PCD, as nasal NO is extremely low in patients with PCD [42, 45, 46].

However, its clinical diagnosis is not complex, as patients may present with symptoms of recurrent respiratory infections. Obtaining information about couples' family history is essential, as the incidence of PCD among offspring of consanguineous marriages is high. Imaging investigations of the chest and sinus may help with the diagnosis. Moreover, it can identify dextrocardia, which is the main basis for obtaining an imaging diagnosis of KS [10].

Infertility Management and Prognosis

The first report correlating PCD with male infertility dates back to the 1970s and was described by Afzelius [12]. However, PCD carriers can be considered fertile in some cases, and this is due to heterogeneous axonema ultrastructural defects, which do not necessarily result in total immobility in the spermatozoa [44]. The uncoordinated movements of the cilia are not always present in spermatozoa, since they have flagella swimmers with different motility patterns. This could explain the paradoxical association between bronchopulmonary disease and normal fertility in some KS patients [2, 43, 44].

When PCD patients face infertility problems, assisted reproduction techniques such as ICSI mean that complete asthenozoospermia is no longer a limitation to fertilization and pregnancy; in fact, fertility levels can be obtained that are comparable to those associated with ICSI with mobile spermatozoa [47]. The major challenge in the use of immobilized spermatozoa for ICSI has been the differentiation between living and dead sperm, for which the hyposmotic test has proven to be quite effective and suitable in the selection of live spermatozoa in these cases. The hyposmotic solution consists of 50% culture medium and 50% deionized water [43, 48–50]. The use of this solution to select viable, but still, spermatozoa for ICSI is a simple and practical method and is associated with acceptable rates of fertilization and pregnancy [51, 52]. Fertilization rates in KS cases with completely immobile sperm have been reported as being between 0% and 75% [51–57]. Aiming to improve sperm motility and sperm selection, it is also possible to use some additional strategies, such as the use of multiple ejaculations before providing a sample for the ICSI procedure, while also using pentoxifylline (a phosphodiesterase inhibitor) to stimulate sperm movement [10].

However, it has also been shown that fertilization may be improved with testicular spermatozoa, even if they are immotile [57]. Several reports have shown that immobile sperm obtained from the testis produce similar or better rates of fertilization and pregnancy than the immobile ejaculate sperm [56, 58, 59]. Testicular spermatozoa for ICSI appear to have a reliable fertilization capacity in men with KS, while ejaculated spermatozoa, even if tested as viable, appear more unpredictable [43]. It has been shown that the fertilization ability of totally immobile testicular sperm is better than that of totally immobile ejaculated sperm. This has been attributed to the long transit time required for sperm to pass through the epididymis. This increases the risk of senescent degeneration of sperm [60]. In reported cases of ICSI with testicular spermatozoa of men with KS, only immobile spermatozoa were found in both the ejaculate and the testicle, and testicular spermatozoa were injected, which were considered viable after the hyposmotic test, with fertilization rates ranging from 53% to 63% [51, 52, 55]. Despite evidence of a better rate of fertilization using testicular spermatozoa, several researchers recommend the use of spermatozoa from repeated ejaculation in men with total asthenozoospermia in order to achieve significantly better viability and fertilization capacity [57, 61]. In a rare case of identifying mobile spermatozoa in the ejaculate of men with KS, the fertilization rate was 73% in the first cycle of stimulation and 100% in the second cycle, suggesting that a high rate of fertilization could be obtained if spermatozoa could be used for ICSI, even in patients with KS [47].

Aberrations of sex chromosomes between the embryos of men with KS are not common; a preimplantation genetic diagnosis may be offered to the couples being treated prior to the transfer of the embryo, and genetic counseling is mandatory for these patients [52]. Genetic counseling is mandatory for couples facing infertility treatments, as the defects associated with PCD may not only cause infertility, but they may also be associated with an increase in the risk of genetic defects in the offspring [10].

Conclusion

Although PCD patients may present with severe infertility due to total asthenozoospermia, the affected males may benefit from assisted reproductive treatments with the use of ICSI, and they may conceive their offspring with their own sperm. It is a rare genetic disease in which mutations reported in known genes were found in about 70% of cases. Therefore, additional genes have yet to be identified.

Review Criteria
A thorough search of medical literature was conducted with the MEDLINE, EMBASE, ScienceDirect and Scielo databases until October 2018. We used relevant terms, namely, "Kartagener syndrome," "immotile cilia syndrome," "primary ciliary dyskinesia," and "male infertility."

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Chapter 17
Persistent Müllerian Duct Syndrome

Robert A. Petrossian and Nicholas N. Tadros

Key Points

- Etiology: persistent Müllerian duct syndrome (PMDS) is caused by an abnormally functioning anti-Müllerian hormone (AMH) pathway in either the AMH hormone, its receptor AMHRII, or idiopathically, leading to the persistence of Müllerian structures (uterus, fallopian tubes, and proximal vagina).
- Incidence: unknown, roughly 300 cases have been reported in the literature.
- Presentation: 46,XY males with three anatomic variants:
  - "Female form": bilateral cryptorchidism with intrapelvic testes in an ovarian-like position.
  - Hernia uteri inguinalis: unilateral cryptorchidism (intrapelvic testis) with contralateral inguinal hernia containing testis along with its Müllerian structures.
  - Transverse testicular ectopia: unilateral cryptorchidism with contralateral inguinal hernia containing both testes along with part of their Müllerian structures.
- Diagnosis: Imaging (MRI preferred) and pathology following laparoscopy.
- Treatment: aim is preventing its two main complications—infertility and malignancy—through laparoscopy with orchidopexy and extirpation of Müllerian remnants.

R. A. Petrossian · N. N. Tadros (✉)
Department of Urology, Southern Illinois University School of Medicine, Springfield, IL, USA

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Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance, is produced by Sertoli cells of the fetal testis and normally causes regression of the Müllerian structures that would otherwise persist in the pathway of normal female development. In addition, testosterone produced by fetal Leydig cells in the testis induces virilization of the internal and external male genital tract. Disorders of sex development (DSD) in the male typically stem from an aberrancy involving testosterone, anti-Müllerian hormone, or both.

Persistent Müllerian duct syndrome (PMDS) is a rare form of male pseudohermaphroditism that is classically seen in patients with normal testosterone function but an abnormally functioning AMH pathway. Less commonly, idiopathic cases with an intact AMH pathway have been recognized [1]. PMDS is phenotypically characterized by the presence of Müllerian structures (uterus, fallopian tubes, proximal vagina) in an otherwise normally virilized 46,XY male.

The first documented case of PMDS is often credited to Nilson in 1939 [2]; however, the earliest documentation of this disorder dates back to 1895 [3].

Epidemiology

PMDS is a rare condition, with approximately 300 cases reported in the literature [4]. The majority of cases are discovered in pediatric patients [5]. While the true incidence of PMDS is unknown, the rate of identified cases has seemed to increase in recent years. Approximately 200 cases of PMDS were reported between 1964 and 2012, whereas 34 cases were reported between 2012 and 2016 [4, 6]. This increased rate may be attributable to greater awareness of the disease, along with the advent of laparoscopy and earlier surgical intervention for cryptorchidism.

Embryology

AMH is part of the TGF-β superfamily and plays an important role in reproduction, hormonogenesis, and sexual differentiation and development [7]. As opposed to other TGF-β polypeptides, it is exclusively expressed by the gonads.

Prior to sexual differentiation in the fetus, both Wolffian (mesonephric) and Müllerian (paramesonephric) ducts are present. Normal male sex differentiation depends on the presence of two hormones: testosterone and AMH. During normal male development, testosterone is produced by fetal Leydig cells starting at 9 weeks of gestation. This preserves Wolffian ducts and stimulates virilization of external genitalia. Additionally, Sertoli cells begin to secrete AMH from 7 weeks of gestation, which then acts on anti-Müllerian hormone receptor, type II (AMHR2), caus-

ing regression of the Müllerian ducts [1]. If, however, the Müllerian ducts fail to regress, the testes remain in the pelvis, usually attached to the broad ligament. This relationship of the gonad to the broad ligament is not as robust as it is in normally developing females. It is this distinction that allows the testes, attached to the fallopian tubes, to partially or fully descend toward the inguinal canal and scrotum [8].

Conversely, the female reproductive tract normally develops in the absence of AMH. In PMDS, failure to either secrete AMH or act on AMHR2 causes the persistence of Müllerian structures in the male.

Genetics

Patients with PMDS are genetically 46,XY males [1, 9].

PMDS is inherited by autosomal recessive transmission via mutations of the AMH gene or the AMHR2 gene [10]. A large study, including molecular testing on 157 families with PMDS, identified AMH pathway mutations in 88% of cases [9]. Interestingly, not all patients with PMDS have mutations in AMH or AMHR2. This subset of PMDS patients with intact AMH and AMHR2 are dubbed "idiopathic" PMDS. It follows that rates of consanguinity are expectedly high: 40% in families with AMH mutations and 33% in families with AMHR2 mutations compared to just 10% in idiopathic cases [9].

AMH is a 145-kDa dimeric glycoprotein that binds to a specific type II serine-threonine kinase transmembrane receptor (AMHR2). The human AMH gene is located on the short arm of chromosome 19, in subbands 13.2 to 13.3, and consists of five exons that are 412, 124, 108, 160, and 856 base pairs in length [11].

The gene for AMHR2 is located on the long arm of chromosome 12q13; it is 82-kDa in size and made of 573 amino acids. Its extracellular domain is the binding site for the ligand, while its intracellular domain carries the serine-threonine kinase activity [1, 12].

Presentation

Patients with PMDS outwardly exhibit a grossly normal male phenotype and are usually assigned a male sex at birth without hesitation [13]. As such, PMDS is often discovered incidentally during surgery for undescended testis or hernia repair [14]. Given the rarity of this condition, the diagnosis can often be missed despite surgical correction of hernia or undescended testis [15]. In adults, patients may present with infertility, hematuria, or hematospermia [9, 13].

PMDS can present in three different forms: bilateral cryptorchidism (also described as the "female form"), unilateral cryptorchidism (termed "hernia uteri inguinalis"), and transverse testicular ectopia [9].

Bilateral cryptorchidism occurs when the testes remain in the pelvis in essentially the normal location of the ovaries. This is the most common presentation and is found in approximately 55% of cases with a mutation in the AMH pathway and 86% of idiopathic cases.

Unilateral cryptorchidism arises when a single testis is in an inguinal hernia along with its ipsilateral fallopian tube and a portion of the uterus. Despite the presence of Müllerian structures in the hernia sac, the diagnosis of PMDS is often missed. This presentation is seen in approximately 20% of AMH pathway mutations and 14% of idiopathic cases.

Transverse testicular ectopia is a well-documented phenomenon associated with PMDS. Here, both testes along with part of their Müllerian structures migrate toward the same hemiscrotum and herniate into a single patent processus vaginalis. An example presentation would be nonpalpable left testis with an undescended right testis and right inguinal hernia [16]. Notably, this presentation is exclusively seen in approximately 25% of those with AMH pathway mutations; i.e., transverse testicular ectopia is never seen in idiopathic cases [9].

Clinical Features

Patients with PMDS externally exhibit a normal male phenotype, making straightforward diagnosis problematic. Careful physical examination will, however, reveal unilateral or bilateral cryptorchidism with a potentially associated inguinal hernia. In postpubertal patients, symptoms may include infertility, oligospermia, hematuria, hematospermia, or inguinal hernia [9, 13, 17].

The size of the descended testis in prepubertal patients is comparable to that of reference curves of normal testes. Furthermore, compensatory testicular growth was greater in prepubertal patients compared to adults, supporting the view that the testicular insult may be related to neglected cryptorchidism rather than an integral component of the syndrome [18].

Most patients with PMDS are azoospermic [16]. This can be attributed to abnormalities of the male excretory ducts seen in PMDS. The vas deferens can be narrowed, blind ending, or even completely absent. Epididymal dissociation from the testis has also been described. In addition, the length of the testicular (internal spermatic) arteries are often diminished, necessitating a division of these vessels to allow scrotal placement of the testes [9]. Even in the absence of these findings, cryptorchidism can contribute to these patient's infertility as well.

It has been estimated that 3.1–8.4% of males with PMDS will develop malignancies in retained Müllerian structures [4]. There have been reports in the literature of clear cell adenocarcinoma of the uterus and uterine adenosarcoma within retained Müllerian remnants [19, 20]. The majority of experts appear to agree that the Müllerian structures should be removed whenever possible to reduce the risk of Müllerian malignancy and to avoid the burden of lifetime follow-up.

The risk of testicular cancer is about 1% in the general male population. On the other hand, cryptorchid patients have a relative risk 3.7–7.5 times higher than those with intrascrotal testes [21]. That said, the risk of testicular cancer in the undescended testes of PMDS patients is similar to that of patients with intraabdominal testes without PMDS, with reported rates of 5–18% [4]. The risk of seminoma is highest in PMDS, as it is in patients without PMDS, although other testicular malignancies (gonadoblastoma, choriocarcinoma, embryonal cell carcinoma, yolk sack tumor, teratoma, mixed germ cell tumor) have been described.[9]

Evaluation and Management

In order to successfully diagnose PMDS, one must have a high degree of awareness and suspicion. Given the rarity of PMDS, there is no standardized algorithm for the evaluation and management of this condition. Nonetheless, there are generally supported expert opinions in the literature. Namely, early diagnosis and intervention is important in the treatment of PMDS, which is primarily aimed at preventing its two main complications: infertility and malignancy.

Diagnosis

Imaging (US and MRI preferred over CT to avoid excess radiation exposure in children) and laparoscopy with pathologic analysis of the removed organs are the two most reliable methods to confirm a diagnosis of PMDS.

Chromosome studies should be considered to confirm the 46,XY karyotype, which is unfailingly seen in PMDS. If a different karyotype is encountered, other diagnoses must be considered. Specific genetic testing for PMDS is an option but can be costly and time-consuming and is generally not recommended.

Enzyme-linked immunosorbent assay (ELISA) testing can be used to assess levels of AMH in order to differentiate between defects in AMH versus AMHR2. Mutations in the AMH gene will result in low or undetectable serum AMH, whereas mutations in the AMH receptor will result in normal or elevated levels of serum AMH. ELISA is thus most useful to delineate the genetic defect once a diagnosis of PMDS is already established. Of note, ELISA is only useful in prepubertal children given that serum AMH expression is suppressed by Sertoli cells under the influence of testicular androgens starting in puberty [1].

Testicular biopsy in prepubescent patients is unnecessary as biopsies have shown normal testicular histology in this population [10]. However, in adults, testicular biopsy may be warranted to assess for dysgenetic or malignant changes.

Differential Diagnosis

In the workup of PMDS, there are a few diagnoses that one must consider.

Mixed gonadal dysgenesis (MGD) is often the most common differential diagnosis to distinguish from PMDS. MGD is a DSD that has a wide variety of presentation and is typically characterized by 45,X/46,XY mosaicism, with both male and female phenotypes [22]. Often there is asymmetric gonadal development with an intraabdominal or dysgenetic testis on one side, a contralateral streak gonad, and persistent Müllerian structures. Ambiguous genitalia can be seen as well. If the patient is phenotypically female or has ambiguous genitalia, then one can rule out PMDS. Otherwise, given that chromosome studies will not show any 45,X karyotype in PMDS, karyotyping is a sensible way to distinguish MGD from PMDS.

Dysgenetic male pseudohermaphroditism (DMP) is a variant of MGD that must also be distinguished from PMDS. In DMP, patients are typically 46,XY and present with bilateral dysgenetic testes, persistent Müllerian structures, cryptorchidism, and inadequate virilization [23]. Thus, if the patient has ambiguous genitalia, PMDS can be excluded.

Inguinal hernia of any abdominal contents (bowel, appendix, bladder, appendix, neoplasms, metastases) is another differential diagnosis to consider. Physical exam can sometimes be unreliable, especially with an occult hernia. Regardless, ultrasound (US) is a safe place to start, though the results may be equivocal. In these situations, magnetic resonance imaging (MRI) has been argued to be the most sensitive, specific, and accurate imaging modality to diagnose inguinal hernias [24].

Fertility

Infertility is the most common complication of PMDS. The risk of infertility is driven by the ectopic testis, as well as abnormal excretory ducts. Orchidopexy simultaneously improves the fertility potential of the testis while reducing the risk of testicular malignancy. Testicular sperm extraction has been described and remains an option for PMDS patients seeking fertility. A comprehensive literature review revealed that 19% of reported adult patients with PMDS had fathered children; interestingly, the vast majority of these patients (save for one) had hernia uteri inguinalis or transverse testicular ectopia [9]. These findings support the notion that fertility is uncommon yet possible as long as there is at least one scrotal testis and patent excretory ducts.

Radiological Studies

From an imaging standpoint, US, MRI, and computed tomography (CT) have all been used to successfully identify persistent Müllerian structures, as well as other

possible related abnormalities, such as transverse testicular ectopia. However, in the pediatric population, one should try to avoid the high doses of radiation associated with CT and consider using US and MRI, which are sufficient to establish a diagnosis of PMDS.

Some characteristic radiological findings have been described depending on the imaging modality utilized and the specific PMDS variant [15]. With transverse testicular ectopia, two testes will be seen in the same hemiscrotum along with Müllerian structures. Conversely, the scrotal contents will be empty in the "female form" of PMDS.

With hernia uteri inguinalis, sagittal scrotal ultrasound images will often reveal a fluid-filled thick-walled structure within the scrotal sac, representing the fluid-filled endometrium of the uterus.

On CT, one will appreciate a blind-ending, fluid-filled tubular structure just posterior to the bladder, which potentially extends down into the inguinal canal and/or scrotum if the hernia uteri inguinalis variant of PMDS is encountered. The ovaries will be characteristically absent, whereas the seminal vesicles may or may not be present. The prostate and ejaculatory ducts will be present, and the latter may be communicating with the upper vagina.

MRI is arguably the best imaging modality to confirm a diagnosis of PMDS, given its excellent ability to delineate complex soft tissue anatomy. MRI will demonstrate a blind-ending, fluid-filled structure with signal characteristics and the zonal anatomy of a uterus and possibly connected to a fluid-filled structure delineating the upper vagina. Of note, the lower third of the vagina will be absent. Ovaries will be characteristically absent, whereas the seminal vesicles may or may not be present. Prostate and ejaculatory duct anatomy will be similar to CT findings.

Surgical Management

Diagnostic laparoscopy is far and away the most accurate approach to the nonpalpable testis [25]. Considering that PMDS inherently involves a nonpalpable testis (i.e., either intraabdominal or in the contralateral hemiscrotum), diagnostic laparoscopy is the preferred surgical approach for the diagnosis and treatment of PMDS. Elective exploratory laparotomy has also been described [15].

For surgical purposes, it is important to note that the male excretory ducts in PMDS are in intimate anatomic relation with the Müllerian structures [9]. Attempts to extirpate Müllerian derivatives will therefore pose a high risk of damage to the epididymis, vas deferens, and deferential artery. If orchidopexy either fails or is technically impossible, then orchiectomy should be reserved strictly as a last option in order to prevent the risk of future testicular malignant transformation [9]. Put another way, orchiectomy should only be performed if streak or dysgenetic gonads are seen, if the testes have undergone malignant change, or the testes cannot be adequately mobilized to a palpable position.

Other Considerations

The physician should provide reassurance to the patient and/or family regarding gender identity. Psychiatric referral and counseling should be offered if deemed appropriate by the physician.

One must also consider a workup in siblings and second-degree relatives of patients with PMDS [14, 26]. Often, scrotal and pelvic US is a good place to start, possibly followed by MRI to better delineate the soft issues within the pelvis if the ultrasound is equivocal. Nonetheless, laparoscopy remains the best way to diagnose and manage PMDS. Genetic counseling may also be considered if the parents are planning on having more children.

If Müllerian or male reproductive structures are left in situ or cannot be removed, lifetime follow-up is recommended, though no standardized follow-up schedule has been established.

Review Criteria

- PubMed search included persistent Müllerian duct syndrome, hernia uteri inguinalis, transverse testicular ectopia ($n = 505$)
  - Articles were screened by title and abstract
  - Individual case reports, animal studies, and studies that were not relevant to the topic at hand were excluded ($n = 483$)
  - There were 22 articles cited from the initial search
- A secondary PubMed search included genes and receptors involved in the anti-Müllerian hormone pathway ($n = 32$)
  - Articles were screened by title and abstract
  - Individual case reports, studies reporting rare or novel mutations, and studies that were not relevant to the topic at hand were excluded ($n = 28$)
  - There were four articles cited from the secondary search
- A total of 26 articles were cited

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Chapter 18
Disorders of Sex Determination

Ibrahim A. Abdel-Hamid, Ezzat S. Elsobky, and Moustafa A. Elsaied

Key Points

- Defects in the genes regulating gonadal determination generally result in defective organ formation and a subset of disease states grouped under the title "disorders of Sex Determination" or DSD.
- Reaching a genetic diagnosis, taking into account the clinical and biochemical phenotypes, has an impact on the provision of medical care and also informs us about future fertility potential.
- Nonsyndromic SRY-positive 46, XX testicular DSD is an excellent demonstration of how DSD elicits male phenotype with infertility, which largely overlaps with the Klinefelter's syndrome.
- The clinical presentation of nonsyndromic 46, XX testicular DSD shows considerable variation ranging from the classic male phenotype to 46, XX true hermaphrodites (also known as 46, XX ovotesticular DSD).
- Patients with 46, XX testicular DSD are unable to father a biologically related child by undergoing assisted reproduction owing to the lack of Y-chromosome-linked azoospermia factor (AZF) regions.

I. A. Abdel-Hamid (✉) · M. A. Elsaied
Department of Andrology, Mansoura Faculty of Medicine, Mansoura University Hospital, Mansoura, Egypt

E. S. Elsobky
Genetics Unit, Pediatrics Department, Ain Shams University, Medical Genetics Center, Cairo, Egypt

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Introduction

Human sex development is a complex biological process that can be divided into three consecutive major steps [1]. The first step occurs at fertilization based on whether one inherits an X or a Y chromosome from one's father (sometimes referred to as "chromosomal sex"). The embryo, regardless of genetic sex (i.e., 46, XX or 46, XY), is bipotential (i.e., sex indifferent) [2]. In the second step (gonadal sex determination, alternatively called primary sex determination), bipotential gonads develop into either testes or ovaries. The process typically occurs at approximately 6–7 weeks of gestation in the human male fetus. It is important to note that ovarian differentiation is delayed until 12 weeks of gestation [2, 3]. The SRY is believed to act as a switch signal for testis differentiation [4, 5]. Additional genes that are both autosomal and X-linked, some located upstream or downstream from the *SRY* gene, are involved in testis development [6, 7]. Some genes promote testis formation, some maintain it, and yet others oppose them. This pathway and its control are still being explored [8]. Each cell type within the bipotential gonad exhibits a dual potential, and as such an expression pattern is orchestrated within each cell type to promote the predetermined phenotype and actively represses the alternative pathway [2, 3]. In short, the biological steps that lead to the formation of the testes have been named "sex determination."

The next and final step is differentiation of the internal and external genitalia (sex differentiation). At this time, the fully formed testes or ovaries secrete local and hormonal factors to induce differentiation of internal and external genitals. Early in pregnancy, all embryos have both paramesonephric ducts (Müllerian ducts) and mesonephric ducts (Wolffian ducts) [9]. If a testis develops, anti-Müllerian hormone (AMH), a glycoprotein secreted by immature Sertoli cells acts on its receptor in the Müllerian ducts inducing their regression [10]. Testosterone secreted by the developing testicular Leydig cells acts on the androgen receptor in the Wolffian ducts, inducing the development of the epididymis, vas deferens, and seminal vesicles. Testosterone is further reduced to dihydrotestosterone (DHT), which acts on androgen receptors to androgenize (masculinize) the external genitalia [9].

Mutations or defects in the genes regulating gonadal determination are generally grouped under the title "disorders of Sex Determination" (Table 18.1). Based on both the 2006 International Consensus Conference on intersex disorders [11] and the 2016 update [12], these disorders of sex determination are now included under the broad categories of disorders of sex development (DSD, also known as differences of sex development). The nomenclature of this group of conditions remains controversial. Isolated forms of DSD—in which symptoms are confined to sex development—can arise when mutations occur in gonad-specific regulatory regions of genes. However, most genes related to sex development are expressed not only in the developing gonad but also in other organs. Therefore, defects in these genes may lead to syndromic DSDs [13].

Disorders of sex determination leading to abnormal gonadal formation include Turner's and Klinefelter's syndromes as the most common genetic syndromes

Table 18.1 Disorders of sex determination

I. *Sex chromosome disorders* (abnormal number of sex chromosomes, also known as sex chromosomal disorders of sex development)	*Klinefelter syndrome* and variants *Turner syndrome* and variants 45, X/46, XY 46, XX/46, XY
II. *46, XY disorders of sex development* (testes fail to undergo normal development)	*Gonadal dysgenesis* (pure, partial, or mixed) 1. *Isolated gonadal dysgenesis (GD)* 2. *Syndromal*: Gonadal dysgenesis with Adrenal hypoplasia syndrome (*SF1/NR5A1* gene defect) Denys–Drash syndrome (*WT-1* gene defect) Frasier syndrome (*WT-1* gene defect) Campomelic dysplasia syndrome (*SOX9* gene defect) GD with neuropathy syndrome *(DHH* gene defect) X-linked alpha thalassemia/mental Retardation syndrome (*XH2* gene defect) Palmoplantar hyperkeratosis with squamous cell carcinoma syndrome (*RSPO1* gene defect) Blepharophimosis-ptosis-epicanthus inversus syndrome type I (*FOXL2* gene defect)
III. *46, XX testicular DSD* (46, XX sex reversal/true hermaphroditism)	A. *Based on SRY+ or SRY−* 1. XX^{Y+} (SRY translocation usually to the terminal Xp or, rarely, an autosome) (80–90%) 2. XX^{Y-} (probable mutation of downstream regulator of testis development) (10–20%) B. *Based on clinical features* 1. Classic XX males, without ambiguous genitalia 2. XX males with ambiguous genitalia 3. XX true hermaphrodites (also known as 46, XX ovotesticular DSD).

involving sex chromosomes. On the other hand, 46, XX testicular DSD is a much less common syndrome. The objectives of this chapter are to (a) present a patient representing disorders of sex determination (nonsyndromic 46, XX testicular DSD); (b) discuss approaches to the diagnosis, the lessons learned, and the management options; and (c) review shortly the genetics of human sex determination.

Case Presentation

A 33-year-old man married for 18 months presented with primary infertility after the female factor had been ruled out as a cause. He had a university degree. The patient had spontaneous testicular descent to scrotum since birth and a timely pubertal growth. He reported decreased libido and erectile dysfunction. His sense of smell was normal. His family history was unremarkable. He had no significant medical or surgical history and was on no regular medication. Physical examina-

tion revealed masculine body contours with lack of temporal recession of hair (Fig. 18.1). The facial, armpit, and pubic hairs were sparse (Figs. 18.1 and 18.2). His height was 161 cm. His upper body segment was 78.5 cm, lower body segment was 82.5 cm, and arm span was 164 cm. His weight was 78 kg. The patient showed moderate gynecomastia bilaterally (Fig. 18.1). He had a normal virilized voice. No skeletal abnormalities were identified by physical examination. The patient had well-developed scrotum and penis with stretched penile length of 12.8 cm. There was no hypospadias. The testes were small and soft with the left testicle measured 1.8 cm × 1.1 cm × 0.8 cm and the right testicle at 2.1 cm × 1.3 cm × 0.9 cm using ultrasound examination. The vas deferens was palpable, and there was no palpable varicocele. An ultrasound of the pelvis showed no evidence of uterus or ovaries.

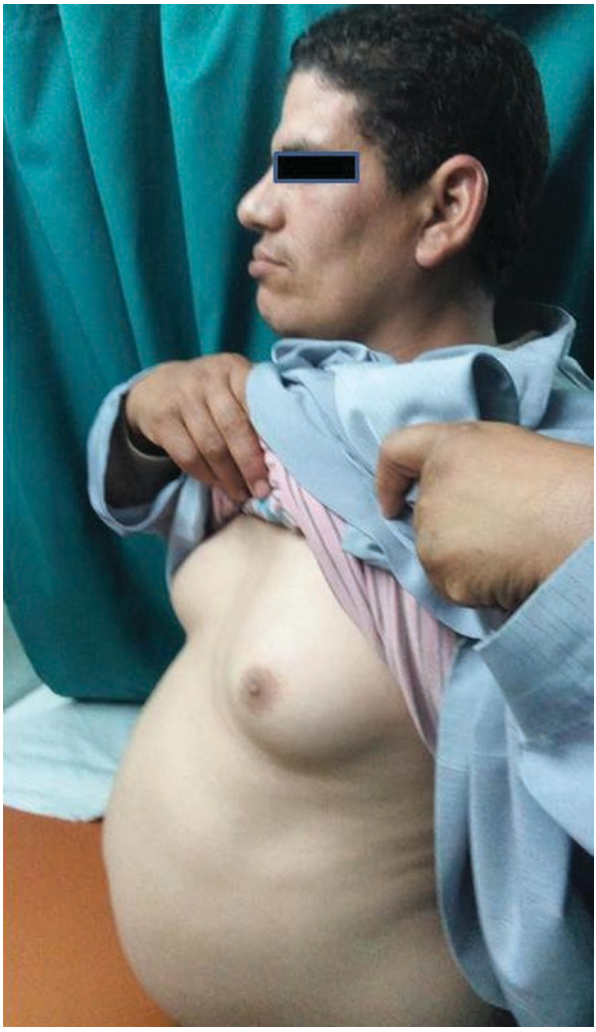

Fig. 18.1 Shows lack of temporal recession of hair, decreased facial hair, and bilateral gynecomastia

Fig. 18.2 Shows sparse pubic hair, well-formed penis, and small testes

Transrectal ultrasonography for internal genitalia showed normal prostate, seminal vesicles, and ejaculatory ducts. Repeated semen analyses demonstrated azoospermia with an average seminal volume of 2 ml. Seminal fructose, viscosity, and pH were normal. Hormonal assay revealed the following: luteinizing hormone (LH), 31 mIU/ml (normal range: 1.5–9.3 mIU/ml); follicle-stimulating hormone (FSH), 42 mIU/ml (normal range: 1.4–18.1 mIU/ml); total testosterone, 201 ng/dl (normal range: 241–827 ng/dl); estradiol, 60.3 pg/ml (normal range: 21–76 pg/ml); and prolactin, 8.1 ng/ml (normal range: 2.1–17.7.0 ng/ml).

How the Diagnosis Was Made

Based on the best available clinical and biochemical profile, a diagnosis of azoospermia (absence of spermatozoa in the seminal fluid) was considered. Azoospermic men with small testicular volume may have either primary testicular failure (primary or hypergonadotropic hypogonadism) or secondary testicular failure (hypogonadotropic hypogonadism). Rarely, hypogonadism can occur in complete (testicular feminization) or partial (Reifenstein's syndrome) androgen insensitivity syndrome [14]. In such cases, as has been noted in our patient, a hormonal evaluation would aid in the differential diagnosis. Primary testicular failure (hypergonadotropic hypogonadism) is strongly suggested in this patient due to the presence of bilateral gynecomastia, decreased libido and erectile dysfunction, and elevated LH and FSH levels accompanied by low serum testosterone levels. Because of diurnal variation, blood samples used to measure testosterone should be taken prior to 10 o'clock in the morning. The Food and Drug Administration (FDA) defines hypogonadism as a testosterone level $\leq$300 ng/dL [15].

Pediatric disorders such as cryptorchidism, orchitis, or testicular torsion and acquired causes such as trauma or medication usage were excluded by negative medical and surgical history. Anorchia, tumors, and varicocele were excluded by physical examination and imaging study. Klinefelter syndrome came to suspicion due to the presence of bilateral gynecomastia, small testicular size, NOA, and elevated LH and FSH levels accompanied by low serum testosterone levels. However, there is no single Klinefelter profile presentation as 47, XXY men can present with any phenotype imaginable, depending on how much circulating testosterone they have, from completely hypogonadal to normally virilized [16, 17]. A karyotype, being the most common genetic analysis required for evaluation of the NOA male, was performed, the result of which showed 46, XX, which was consistent with the normal female karyotype (Fig. 18.3). Consequently, fluorescence in situ hybridization (FISH) analysis was performed and showed that SRY locus had been translocated to the short (p) arm of the X chromosome (Fig. 18.4). The diagnosis of a nonsyndromic SRY-positive 46, XX testicular DSD is established in this patient based on the above clinical features; 46, XX chromosomal complement; and FISH findings.

Nonsyndromic 46, XX testicular DSD (also known as 46, XX male syndrome; XX male; 46, XX sex reversal 1; or De la Chapelle syndrome) is a genetic disease in which a phenotypic male has a female genotype. Our patient is notable as a rare example of disorders of sex determination occurring in about one in 20,000 newborn males (according to Genetics Home Reference, a service of the National Library of Medicine that is part of the NIH) and representing 0.2% of infertile patients [18, 19]. The key lessons that could be learned from this case study may include the following: (a) approaches to an azoospermic patient vary; (b) there are some disorders that come to suspicion in the differential diagnosis (Table 18.2); (c) the clinical presentation of nonsyndromic 46, XX testicular DSD shows considerable variation; (d) genetic testing is carried out not only to detect men in whom

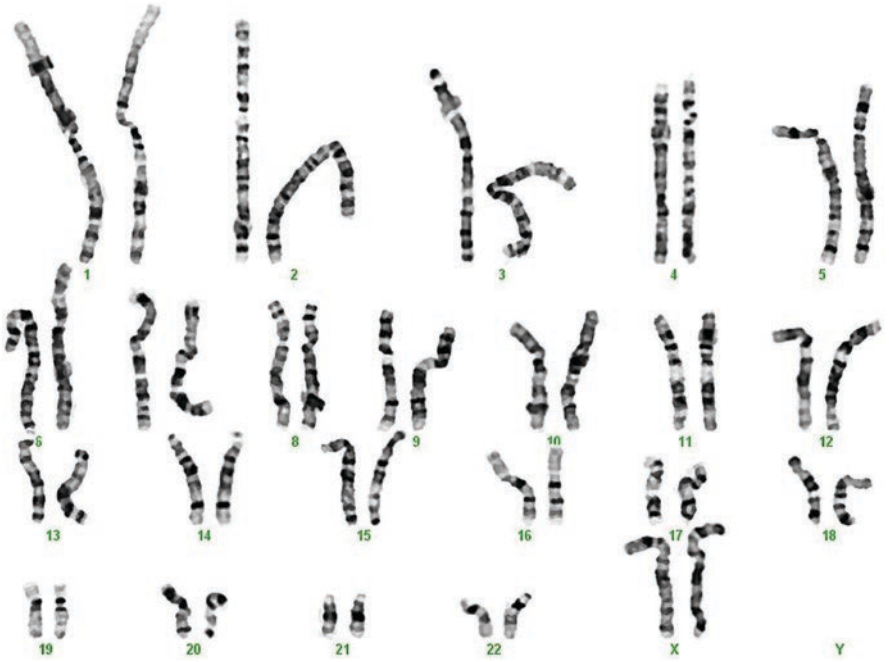

Fig. 18.3 Shows karyotype analysis of the patient (46, XX)

NOA is caused by genetic abnormalities but also to counsel the affected patients about their future fertility potential; and (e) seeking fertility treatment is not always fruitful in some cases of NOA.

Genetic and Epigenetic Pathways of Sex Determination

Human sex determination is a tightly controlled and highly complex process where the bipotential gonad anlage develops as an ovary or a testis. The process involves a complex series of events with a large number of genes and networks acting synergistically or antagonistically and requires a delicate dosage balance in the timing and levels of expression of several genes [20, 21]. Given the complexity of these processes, it has become clear that male and female sex development is achieved through the repression of the alternative state. In other words, a gene determining the formation of a testis may function by repressing the female state and vice versa [20, 22–24]. Generally, factors involved in male sexual determination have been well studied; in contrast, the pathways regulating female sexual differentiation remain incompletely defined. Adding to the complexity of these processes are epigenetic changes (DNA methylation, histone modifications, and noncoding RNAs) that may alter the microenvironment for the multitude of DNA-binding fac-

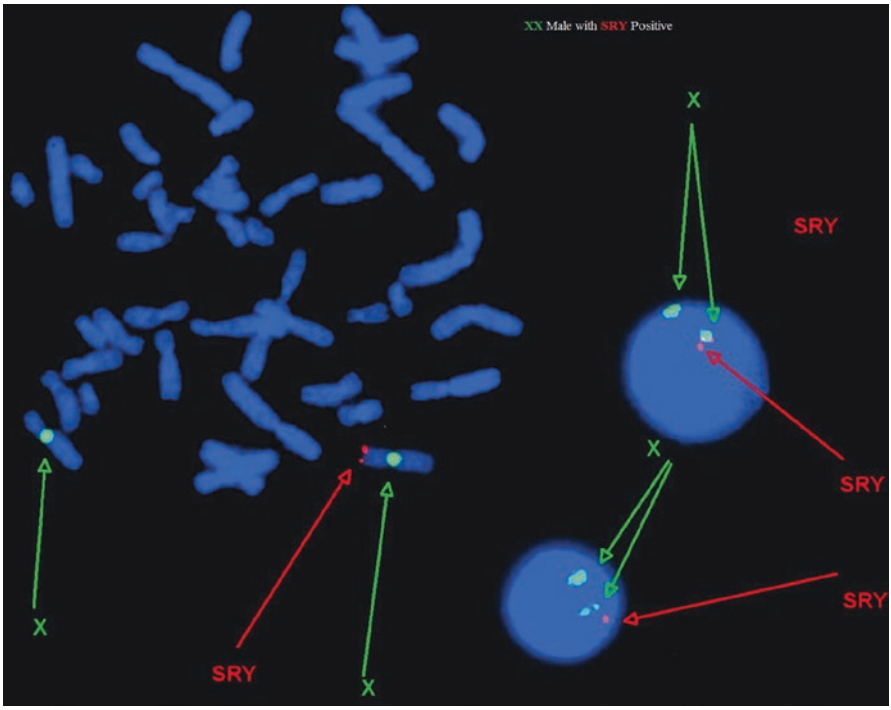

Fig. 18.4 Fluorescent in situ hybridization (FISH) on metaphase chromosomes of the patient using centromeric X (spectrum green), SRY unique sequence probe (red), and DAPI (4′,6-diamidino-2-phenylindole) counterstaining (blue). Metaphase spread showing two X centromeric green signals and one SRY red signal on the short arm of one X chromosome

tors regulating gene expression [25–27]. In humans, changes in this delicate balance between male and female pathways can lead to DSD.

SRY is well established as the primary testis-determining gene that induces expression in the Sertoli cell precursors initiating testis determination by activating downstream effectors such as *SOX9* (*SRY*-related high-mobility group [HMG]-box 9) [4, 22]. This will initiate a complicated cascade of genetic networks that mediate testis determination and differentiation [28, 29]. Other genes are also postulated to be involved in the process of sex determination either upstream or downstream of SRY (*see* Table 18.3). In the absence of SRY in the female fetus, ovary-specific transcription factors, namely forkhead transcription factor 2 (FOXL2); wingless-type MMTV integration site family, member 4 (WNT4); R-spondin 1 (RSPO1); and the activated b-catenin pathway, initiate and maintain ovarian differentiation [30, 31]. The molecular basis of the opposition between male (SRY–SOX9–FGF9) and female (RSPO1–WNT4–FOXL2) pathways is not well understood, and much research remains to be done to delineate all the points of interaction between these pathways**.**

Table 18.2 Differential diagnosis of 46, XX testicular DSD

Differential diagnosis	Prominent differentiating features
1. Nonsyndromic SRY-positive 46, XX testicular DSD	Presents after puberty Shorter-than-average stature Gynecomastia Small soft testes may be firm with age Nonobstructive azoospermia Symptoms of testosterone deficiency Hypergonadotropic hypogonadism secondary to testicular failure No evidence of Müllerian structures Confirmed by karyotype analysis and FISH findings
2. Nonsyndromic SRY-negative 46, XX testicular DSD	Tends to present with ambiguous genitalia at birth, such as penoscrotal hypospadias and cryptorchidism Shows gynecomastia around the time of puberty Confirmed by karyotype analysis, FISH findings, and other molecular genetic testing
3. Syndromic XX testicular DSD	Associated nongenital manifestations may include (a) Palmoplantar keratosis (R-spondin 1 defect) (b) Microphthalmia and linear skin defects (microdeletion of Xp) (c) Facial dysmorphic features (translocation involving the SOX9 region) (d) Developmental delay (genetic defect around *SOX3*)
4. Klinefelter's syndrome	Usually normal or tall stature with eunuchoidal body proportions Signs and symptoms of hypergonadotropic hypogonadism Small firm testes Gynecomastia Nonobstructive azoospermia Speech delay, learning disorders, and behavioral problems Confirmed by karyotype, 47, XXY and its variants (48, XXXY; 49, XXXXY, and 46, XY/47, XXY mosaicism)
5. 46, XX/46, XY	External genitalia ranging from typical male to ambiguous to typical female Confirmed by karyotype analysis
6. 45, X/46, XY	Male phenotype May have short stature depending on the percentage of 45, X cells If the percentage of 45, X cells is very high, the phenotype is likely to be female with classic Turner syndrome Clinically similar to 46, XX testicular DSD Confirmed by karyotype
7. Autosomal XX sex reversal caused by duplication of SOX9	May present in infancy as severe penile/scrotal hypospadias or normal external and internal male genitalia with small testes In adult, shows infertility with atrophic testes, NOA, and Sertoli-cell-only syndrome Karyotype analysis shows 46, XX The presence of SRY should be ruled out by FISH Confirmed by polymerase chain reaction examination to demonstrate duplication of SOX9

(continued)

Table 18.2 (continued)

Differential diagnosis	Prominent differentiating features
8. 46, XX ovotesticular DSD	The phenotype varies from male to female 75% grow up as males, most of them show chordee, hypospadias, cryptorchidism, and gynecomastia Normal male phenotype does not exceed 10% Considered in all newborns with genital ambiguity and asymmetry of external genitalia/gonads or inguinal hernia Suspected in those children who present with genital ambiguity and bilateral nonpalpable gonads, after excluding CAH due to 21α-hydroxylase deficiency Infertility is the complaint in some patients Most of the patients have a urogenital sinus remnant s in varying positions Some patients consult because of an irreducible hernia that may contain a uterus Gonads may be bilateral or unilateral and may present anywhere along the route of testicular descent from abdomen to labioscrotal fold. Karyotype: 46, XX (70% of cases); 46, XX/46, XY (20%); and 46, XY (10%) The diagnosis should be established histopathologically by documenting the ovotestis.

Table 18.3 Genes involved in human sex determination

Name/ gene ID	Location	Description	Function(s) in sex determination process	Human clinical phenotype of gene abnormalities (reference)
Genes involved in the development of the bipotential gonad (upstream to SRY)				
EMX2 ID: 2018	Chromosome 10, NC_000010.11 (117542445..117549546)	Empty spiracles homeobox 2 [Homo sapiens (human)]	Proposed to function in dorsal telencephalon, olfactory neuroepithelium Development of the urogenital ridge	46, XY DSD; single kidney; intellectual disability; schizencephaly [43] 46, XX female Müllerian duct anomalies [44]
LHX9 ID: 56956	Chromosome 1, NC_000001.11 (197912505..197935476)	LIM homeobox 9 [*Homo sapiens* (human)]	Protein–protein interactions in gonadal development	No human reports linking the gene with DSD

Table 18.3 (continued)

Name/ gene ID	Location	Description	Function(s) in sex determination process	Human clinical phenotype of gene abnormalities (reference)
NR5A1 ID: 2516	Chromosome 9, NC_000009.12 (124481236..124507420, complement)	Nuclear receptor subfamily 5 group A member 1 [*Homo sapiens* (human)]	Regulates the transcription of numerous genes that are expressed in hypothalamic-pituitary-gonadal axis and adrenal cortex that coordinate gonadal development, steroidogenesis, and sex differentiation	46, XY disorders of sex development gonadal dysgenesis with adrenal insufficiency [45, 46] 46, XX primary ovarian insufficiency [47, 48]
WT1 ID: 7490	Chromosome 11, NC_000011.10 (32387775..32435539, complement)	Wilms tumor 1 [*Homo sapiens*(human)]	Activation of SRY Role in the normal development of the urogenital system	46, XX testicular disorder of sex development [49] Denys-Drash syndrome and Frasier syndrome with 46, XY female sex reversal [50]
GATA4 ID: 2626	Chromosome 8, NC_000008.11 (11676919..11760002)	GATA-binding protein 4 [*Homo sapiens* (human)]	Regulation of Sertoli cell function Gonadal functions and development	46, XY gonadal dysgenesis with or without cardiac defect [51, 52]
CBX2 ID: 84733	Chromosome 17, NC_000017.11 (79776254..79787650)	Chromobox homolog 2 [*Homo sapiens* (human)]	Regulates transcription by changing chromatin status Regulates *SRY* expression Stimulates the male pathway and concurrently inhibits the female pathway	46, XY gonadal dysgenesis [53–55]
MAP3K1 ID: 4214	Chromosome 5, NC_000005.10 (56815073..56896152)	Mitogen-activated protein kinase 1 [*Homo sapiens* (human)]	Regulates SRY Expression	46, XY DSD with partial or complete gonadal dysgenesis [56]

(continued)

Table 18.3 (continued)

Name/ gene ID	Location	Description	Function(s) in sex determination process	Human clinical phenotype of gene abnormalities (reference)
ZFPM2 (FOG2) ID: 23414	Chromosome 8, NC_000008.11 (105318859..105804539)	Zinc finger protein, FOG family member 2 [*Homo sapiens* (human)]	Regulation of Sertoli cell function Gonadal functions and development	Familial forms of 46, XY DSD and anorchia [57] 46, XY gonadal dysgenesis [51]
Genes involved in sex determination and testis development				
SRY ID: 6736	Chromosome Yp11.3, NC_000024.10 (2786855..2787741, complement)	Sex determining region Y [*Homo sapiens* (human)]	Initiates male sex determination Induces SOX9 expression	46, XY ovarian DSD with gonadal dysgenesis (loss of function) [58, 59] XX male syndrome (translocation) [32, 35, 37]
SOX9 ID: 6662	Chromosome 17, NC_000017.11 (72121020..72126420)	SRY-box 9 [*Homo sapiens* (human)]	Induces Sertoli cell differentiation Regulates transcription of the anti-Müllerian hormone with SF1	46, XY or 46, XX DSD with gonadal dysgenesis with and without Campomelic dysplasia [60–62] 46, testicular DSD (duplications/ triplications) [63]
SOX8 ID: 30812	Chromosome 16, NC_000016.10 (981808..986979)	SRY-box 8 [*Homo sapiens* (human)]	Has a role in testis cord formation Induces the activities of the AMH promoter and TESCO Consolidates Sox9 function with SF1	Spectrum of phenotypes, including 46, XY DSD; male infertility; and 46, XX primary ovarian insufficiency [64]
SOX3 ID: 6658	Chromosome X, NC_000023.11 (140502987..140505060, complement)	SRY-box 3 [*Homo sapiens*(human)]	Essential for gametogenesis and gonadal function Involved in the determination of cell fate	Duplications (including *SOX3*) and deletion upstream of *SOX3*: XX testicular DSD [65]
SOX10 ID: 6663	Chromosome 22, NC_000022.11 (37972312..37984532, complement)	SRY-box 10 [*Homo sapiens*(human)]	Consolidates Sox9 function with SF1 Induces the activities of the AMH promoter	Overexpression of the *SOX10* gene at 22q13 causes 46, XX sex reversal with other anomalies [66, 67]

Table 18.3 (continued)

Name/ gene ID	Location	Description	Function(s) in sex determination process	Human clinical phenotype of gene abnormalities (reference)
FGF9 ID: 2254	Chromosome 13, NC_000013.11 (21671076..21704501)	Fibroblast growth factor 9 [*Homo sapiens* (human)]	The primary role of FGF9 in the XY gonad is to repress the antitestis activity of WNT4; when WNT4 is absent, FGF9 is not required for SRY/SOX9 to initiate and maintain testis development	6, XX male syndrome SRY-negative with FGF9 gene duplication [68]
NR0B1 (DAX1) ID: 190	Chromosome X, NC_000023.11 (30304422..30309378, complement)	Nuclear receptor subfamily 0 group B member 1 [*Homo sapiens* (human)]	Functions as an antitestis gene by acting antagonistically to SRY	Mutations lead to X-linked adrenal hypoplasia congenita, hypogonadotropic hypogonadism at puberty, and nonobstructive azoospermia [69, 70] Duplication of NR0B1 gene leads to 46, XY dosage-sensitive male-to-female sex reversal [71]
DMRT1 ID: 1761	Chromosome 9, NC_000009.12 (841647..969090)	Double sex and mab-3-related transcription factor 1 [*Homo sapiens* (human)]	Part of the network of sex determination Sertoli cell proliferation and differentiation Coexpressed with SRY in the genital ridge of the male embryo	46, XY partial gonadal dysgenesis [72] Nonobstructive azoospermia [73]
DHH ID: 50846	Chromosome 12, NC_000012.12 (49086656..49094819, complement)	Desert hedgehog [*Homo sapiens* (human)]	Role in regulating morphogenesis	46, XY partial or complete gonadal dysgenesis [74, 75]

(continued)

Table 18.3 (continued)

Name/ gene ID	Location	Description	Function(s) in sex determination process	Human clinical phenotype of gene abnormalities (reference)
Genes involved in ovarian development				
RSPO1 ID: 284654	Chromosome 1, NC_000001.11 (37611350..37634923, complement)	R-spondin 1 [*Homo sapiens* (human)]	Regulates the Wnt signaling pathway activation of the β-catenin signaling pathway granulosa cell differentiation	Familial 46, testicular and ovotesticular DSD [76] Syndromic true hermaphroditism [77]
CTNNB1 ID: 1499	Chromosome 3, NC_000003.12 (41199422..41240453)	Catenin beta 1 [*Homo sapiens* (human)]	Inhibits SRY-SOX9-FGF9 pathway	No human reports of CTNNB1 mutations in DSD
FOXL2 ID: 668	Chromosome 3, NC_000003.12 (138944224..138947140, complement)	Forkhead box L2 [*Homo sapiens* (human)]	Role in the development of the granulosa cells of the ovary and the gonadotropic cells of the anterior pituitary	Blepharophimosis syndrome and premature ovarian failure [78]
WNT4 ID: 54361	Chromosome 1, NC_000001.11 (22117305..22143981, complement)	Wnt family member 4 [*Homo sapiens* (human)]	Regulates cell fate and patterning during embryogenesis Antagonizes the testis-determining factor	Loss of function mutation: XX Müllerian duct agenesis [79] Duplication of 1p (including WNT4 and RSPO1): 46, XY gonadal dysgenesis [80]

About half of DSD cannot be explained at the molecular level yet, suggesting the existence of a number of unknown sex-determining genes. For example, on the one hand, a translocation of *SRY* to the X-chromosome or an autosome could explain the pathogenesis in 80–90% among 46, XX testicular DSD [18, 19, 32]; on the other hand, abnormalities in other genes can probably explain about 10–20% of patients who are SRY negative, such as duplication of SOX9 resulting in 46, XX testicular DSD despite the absence of SRY [33, 34].

Clinical Presentation of Nonsyndromic 46, XX Testicular DSD

Generally, nonsyndromic 46, XX testicular DSD presents as one of these three phenotypes: (i) classic 46, XX testicular DSD, which shows infertility with normal male internal and external genitalia; (ii) 46, XX testicular DSD with ambiguous genitalia,

usually detected at birth by external genital ambiguities, including hypospadias, micropenis, or hyperclitoridy; and (iii) 46, XX ovotesticular DSD (true hermaphrodites), which exhibit internal or external genital ambiguities detected at birth [32, 35–38]. These cases are further classified into two major groups. One group (SRY+) is positive for SRY (about 80–90% of cases) because of the translocation of the SRY gene to another location, which is usually the X chromosome or, rarely, an autosome. This group is often associated with the presence of normal male external genitalia. The other group includes SRY-negative 46, XX (SRY−) males (10–20%), which is more often associated with ambiguous genitalia [35, 38]. In this context, it has been suggested that the variation in phenotype is primarily dependent on two mechanisms: X chromosome inactivation (XCI) pattern and the amount of Y material, including *SRY* gene, that has been translocated to the X chromosome [39].

Management of Nonsyndromic 46, XX Testicular DSD

The distinction between OA and NOA is important to establish that azoospermia is due to spermatogenic failure. NOA is associated with a spectrum of many severe and untreatable conditions associated with an intrinsic testicular impairment (vide supra). Therefore, a detailed medical history and physical examination should be obtained in all azoospermic patients to identify those with NOA. Typical criteria for NOA, including small testicular volume, normal epididymides, normal seminal vesicles and ejaculatory ducts, palpable vasa deferentia, and elevated gonadotropin levels, were demonstrated in this patient. The differential diagnoses to be considered with this presentation are shown in Table 18.2. It is critical to differentiate between all possible causes as their potential outcomes differ, thus affecting patient's management. The possibility of nonsyndromic 46, XX testicular DSD is considered in view of the karyotype analysis, FISH findings, and the absence of nongenital associations. Typically, genetic testing is recommended in all men with NOA. However, all genetic testing options should not be completed for every azoospermic patient. For men with small testis volume and high FSH, karyotype analysis is an important first-step genetic testing. Other specific genetic tests may be warranted depending on the clinical context of NOA and the results of karyotype analysis. Karyotypic abnormalities are identified in 14–19% of men with NOA [40]. 46, XX testicular DSD is a rare karyotype identified in azoospermic men.

Most leading guidelines [11, 41, 42] offer the following approaches for this patient: (a) genetic counseling to provide the patient and family with information on the nature, inheritance, and implications of this genetic disorder to help them make informed medical and personal decision, as well as to provide psychological support. In this context, *SRY*-positive 46, XX testicular DSD is generally not inherited because it results from de novo abnormal interchange between the Y chromosome and the X chromosome, resulting in the presence of *SRY* on the X chromosome and infertility. (b) inform the patient that he is unable to father a biologically related child by undergoing assisted reproduction owing to the lack of Y-chromosome-linked azoospermia factor (AZF) regions, meaning that focal sperm production in the testis is not possible

[11, 32, 35, 37] and his testes will show Sertoli-cell-only pathology on biopsy. (c) treat the patient with testosterone (T) replacement therapy to correct the hypogonadal symptoms, improve the secondary sex characters, and ensure normal development of bone and muscle mass. Discussion about different forms of testosterone therapy and their possible side effects should be considered after diagnosis. (d) bone density scan, dual-energy X-ray absorptiometry (DEXA), should be performed to examine for osteopenia or frank osteoporosis. Depending on the degree of osteopenia, treatment may include calcium, exercise, vitamin D, biphosphonates, or calcitonin. Referral to an internist or endocrinologist may be recommended. (e) in this hypogonadal patient, treatment with T may lead to regression of gynecomastia, although in some patients testosterone may get aromatized to E2, resulting in further breast enlargement. If regression does not occur, and if it causes psychological distress, pain, or tenderness to the individual, reduction mammoplasty can be offered. (f) long-term follow-up monitoring of the patient should be conducted to look for bone densitometry (DEXA) once a year, as well as monitoring of T therapy.

Conclusions

Reaching a genetic diagnosis, taking into account the clinical and biochemical phenotypes, has a significant impact on the provision of medical care and also informs us about future fertility potential. This case is an excellent demonstration of how DSD elicits male phenotype with infertility, which largely overlaps with Klinefelter's syndrome but differs due to lack of Y chromosome. Many cases of DSD present with male infertility. Furthermore, infertility is the most common reason for diagnosis in 46, XX testicular DSD. The condition is often associated with hypergonadotropic hypogonadism. This clinical phenotype could be treated with T therapy to ensure proper masculinization with normal development of bone and muscle mass and to diminish the risk of diseases associated with hypogonadism. It is conceivable that patients with 46, XX testicular DSD are unable to father a biologically related child by undergoing assisted reproduction owing to the lack of Y-chromosome-linked azoospermia factor (AZF) regions, meaning that focal sperm production in the testis is not possible.

Review Criteria

- The objectives of this chapter are to present a patient representing disorders of sex determination (nonsyndromic 46, XX testicular disorders of sex development); discuss approaches to the diagnosis, the lessons learned, and the management options; and review shortly the genetics of human sex determination.
- The authors searched electronic databases from 1966 to October 2018, including PubMed, MEDLINE, EMBASE, EBCSO Academic Search Complete, and Google Scholar using the following keywords: azoospermia; sex determination; 46, XX testicular disorder of sex development; XX male syndrome; testis; small testicular size.

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Chapter 19
Endocrine Genetic Defects

Joseph Thomas Mahon and Nicholas N. Tadros

Key Points

- Male fertility is dependent on an intact hypothalamic-pituitary-gonadal axis influencing both Leydig and Sertoli cells.
- Deviations from either normal Leydig cell or Sertoli cell function may lead to disruption in sperm production.
- Isolated gonadotropic-releasing hormone deficiency and Kallmann syndrome can be distinguished by the presence or absence of the anosmia from the symptomatology.
- Klinefelter syndrome leads to seminiferous tubule hyalinization and fibrosis in the setting of excess gonadotropins.
- Retrieval of sperm may be possible with the replacement of absent gonadotropins.

Genetic Defects of the Testicular Microenvironment

According to the World Health Organization, approximately 15% of couples will be affected by infertility [1]. Male-factor abnormalities will be found in 50–60% of these couples, though in only 20% will male factor prove the sole culprit. Anatomical,

J. T. Mahon
Genitourinary Reconstruction & Men's Pelvic Health, Department of Urology, Loyola University Medical Center, Maywood, IL, USA

N. N. Tadros (✉)
Department of Urology, Southern Illinois University School of Medicine, Springfield, IL, USA

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historical, and hormonal etiologies of male-factor infertility have been identified. While some of these abnormalities may prove reversible, others will not. Male reproduction depends on hormonal influence of testis from the pituitary gland and, in turn, the hypothalamus. This hypothalamic-pituitary-gonadal axis is the engine of male sexual maturation and spermatogenesis.

The hypothalamus receives neuronal input from several areas of the central nervous system, then coalesces these signals to generate a pulse to the pituitary gland via both neuronal pathways and a portal vascular network distinct from the systemic circulation [2]. The hypothalamus produces a 10-amino acid peptide, gonadotropin-releasing hormone (GnRH), which acts on the anterior pituitary gland to promote the secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the systemic circulation. Both LH and FSH are glycoproteins composed of two polypeptide chain subunits, designated α and β. The α-subunit is common to both LH and FSH, as well as many other pituitary hormones; the β-subunit, however, is unique.

It is these gonadotropins that regulate testicular function via their effect on the constituent Leydig and Sertoli cells. LH stimulates Leydig cell conversion of cholesterol to androgens, thereby creating a testosterone-rich testicular microenvironment, while FSH influences Sertoli cells in the growth and nurturing of seminiferous tubules and the resultant spermatogenesis. Homeostasis of these processes is maintained through inhibitory feedback loops to both the hypothalamus and pituitary gland. Maintenance of effective and efficient spermatogenesis and androgenesis is dependent on a harmonious relationship between both Leydig and Sertoli cell functions. Indeed, alterations in one aspect of gonadal function may affect all processes, as depicted in Fig. 19.1. While much remains to be discovered in the realm of male-factor fertility, we will discuss here maladies in the endocrine milieu that are responsible for a successful spermatogenesis and their underlying genetic aberrations. Here we will discuss the entities that have been most studied and thus may aid the practicing clinician. While there exists a multitude of additional identified genetic defects, they represent incompletely understood processes and are such rare disease entities that they have been omitted from the following text. A.

Genetic Defects of LH and FSH

Prader-Willi Syndrome

Prader-Willi syndrome was first described by Prader, Labhart, and Willi in 1956 [3]. Children with Prader-Willi exhibit hypogonadism, delayed/absent puberty, cryptorchidism, neonatal hypotonia, hyperphagia, obesity, shortened stature, and cognitive difficulties [4]. The incidence of the syndrome is estimated at one in 26,000 live births [5].

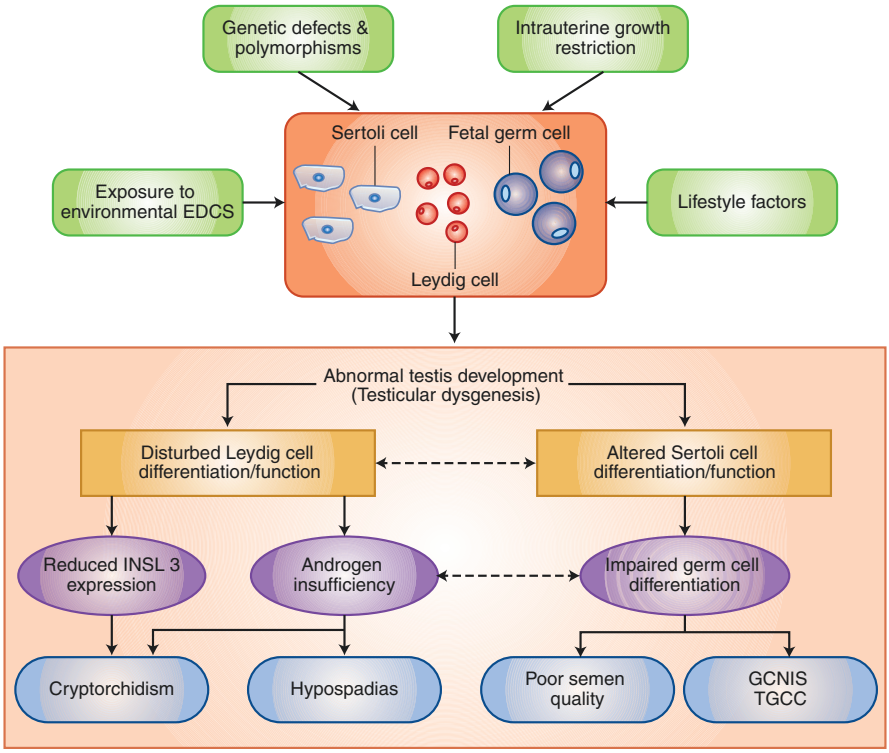


Fig. 19.1 Influence on Leydig and Sertoli cells and their downstream effects

Chromosomal abnormalities of the long arm of paternal chromosome 15 are responsible for the manifestation of Prader-Willi syndrome [6]. A number of defects of genetic coding may lead to the loss of function of 15q. Paternal deletion of the critical region of 15q accounts for the majority of cases of Prader-Willi syndrome, and the variability of these deletions typically escape routine prenatal genetic analysis. Maternal uniparental disomy (UPD) occurs when an offspring inherits both chromosome 15 copies from his mother, and no paternal genetic material is inherited. To a much lesser extent, small genetic mutations (imprinting mutations) or chromosomal translocation may lead to the lack of active paternal chromosome 15q. In all of these scenarios, the lack of expression of paternal chromosome 15q leads to the phenotypic expression of Prader-Willi syndrome [7].

Hypogonadism is most often attributed to hypothalamic dysfunction leading to low LH. Multiple different phenotypes can be observed with the various chromosome aberrancies; cryptorchidism is common and, to a lesser extent, scrotal hypoplasia, microphallus, testis hypotrophy, delayed or incomplete puberty, and infertility. With so many chromosomal abnormalities associated with Prader-Willi syndrome, varying fertility status may be seen among this population.

Though no reports of men with Prader-Willi syndrome fathering children are currently available, a number of small studies have reported on testicular histopathology [8–10]. In a 2008 study, Vogels and colleagues looked at the testicular histology of eight prepubertal boys and one 27-year-old man with Prader-Willi syndrome who underwent either orchidopexy or orchiectomy for cryptorchidism [11]. Testicular histology ranged from normal to Sertoli-cell-only syndrome. The authors extrapolated that fertility phenotype in these individuals may also represent a spectrum, from normal to infertile. Furthermore, with hypogonadism serving as a central feature in Prader-Willi syndrome, it is possible that decreased libido in these individuals is to blame for their lack of paternity.

Though the exact mechanism (and their fertility status in general) has not been completely elucidated in Prader-Willi syndrome, known alteration in hypothalamic function with a resultant LH deficiency is an active component in the infertility/subfertility of the Prader-Will syndrome patient.

Isolated LH Deficiency

Isolated LH deficiency arises from mutations of the β-subunit of the heterodimeric glycoprotein. The gene sequence for the LH β-subunit is located on chromosome 19p13.32. Though the disease has proven exceedingly rare, reported cases have identified multiple mutations leading to the deficiency. Weiss and colleagues (1992) reported on a 17-year-old boy with delayed puberty, absent Leydig cells, and arrested spermatogenesis [12]. Genetic analysis identified a missense mutation substituting arginine for glutamine at position 54 (Gln54Arg) of the LH β-subunit gene. A second report by Valdes-Socin and colleagues (2004) identified a missense mutation substituting aspartic acid for glycine at position 36 (Gly36Asp) [13]. Both of these mutations rendered LH biologically inactive by structurally impairing LH-LH receptor binding and alpha-beta heterodimerization, respectively.

In the normal hypothalamic-pituitary-testis axis, LH stimulates Leydig cells to produce testosterone. Leydig cells produce testosterone levels hundreds of times greater than that of the serum [14]. The testosterone-rich intratesticular microenvironment supports the process of spermatogenesis. Administration of exogenous testosterone increases circulating androgen levels; however, this provides feedback inhibition on the hypothalamic-pituitary-testis axis, thereby decreasing LH secretion and, subsequently, Leydig cell intratesticular testosterone production. Additionally, the testicular microenvironment is shielded from the systemic circulation by the blood–testis barrier. Maintenance of this barrier therefore prevents exogenous testosterone from supporting the testicular microenvironment. Thus, the end result of exogenous testosterone is the depletion of intratesticular testosterone and impaired spermatogenesis, demonstrating the necessity of both FSH and LH for spermatogenesis [15, 16]. In a similar process, loss of LH's influence on the testicular microenvironment via genetic mutation leads to a similar impairment of fertility.

In a 2007 evaluation of two brothers with isolated luteinizing hormone deficiency [17], both men presented with a lack of pubertal development and responded to exogenous testicular therapy. A testicular biopsy specimen from the younger brother revealed interstitial fibrous thickening, hypoplastic seminiferous tubules with a predominance of Sertoli cells, spermatogenic arrest, and absent Leydig cells, supporting the importance of Leydig cells in spermatogenesis. Similar findings have been reported in others with isolated LH deficiency [12, 13, 16]. Both subjects exhibited testicular volume growth after administration of exogenous testosterone. The authors postulated that the proliferation of Sertoli cells due to the synergistic influence of testosterone and elevated FSH was responsible for the testicular growth.

Reversal of isolated luteinizing hormone deficiency has been achieved with use of long-term human chorionic gonadotropin treatment, an analog of LH [18]. The restoration of spermatogenesis appears to vary from individual to individual, with some men exhibiting mature sperm within the ejaculate, while only immature sperm are found in others. Nevertheless, there is hope for paternity among these men. Though no pregnancies have been reported in the literature, it is believed that through the use of ART, men suffering from isolated luteinizing hormone deficiency may be able to father children.

Isolated FSH Deficiency

As opposed to isolated LH deficiency patients, men who suffer from an isolated FSH deficiency exhibit normal androgenization since their pituitary-Leydig cell axis is maintained. However, in the absence of FSH influence on Sertoli cells, spermatogenesis is impaired.

The defect is specific to the FSH ß-subunit gene, located on the short arm of chromosome 11. This exceedingly rare mutation demonstrates an autosomal recessive inheritance pattern. Berger and colleagues (2005) reviewed three previously reported cases of men with isolated FSH ß-subunit mutations [19]. These men all displayed small soft testes, azoospermia, and infertility. In the two men in which genotyping was performed, both were identified homozygosity for a missense mutation for the FSH ß-subunit gene. While two of the men exhibited normal testosterone levels, the third presented with an absence of pubertal development and low testosterone despite high LH levels. Pathologic examination of the testis in isolated FSH deficiency patients reveals narrowed seminiferous tubules, reduced Sertoli cell numbers, absence of germ cells, and Leydig cell hyperplasia [20, 21].

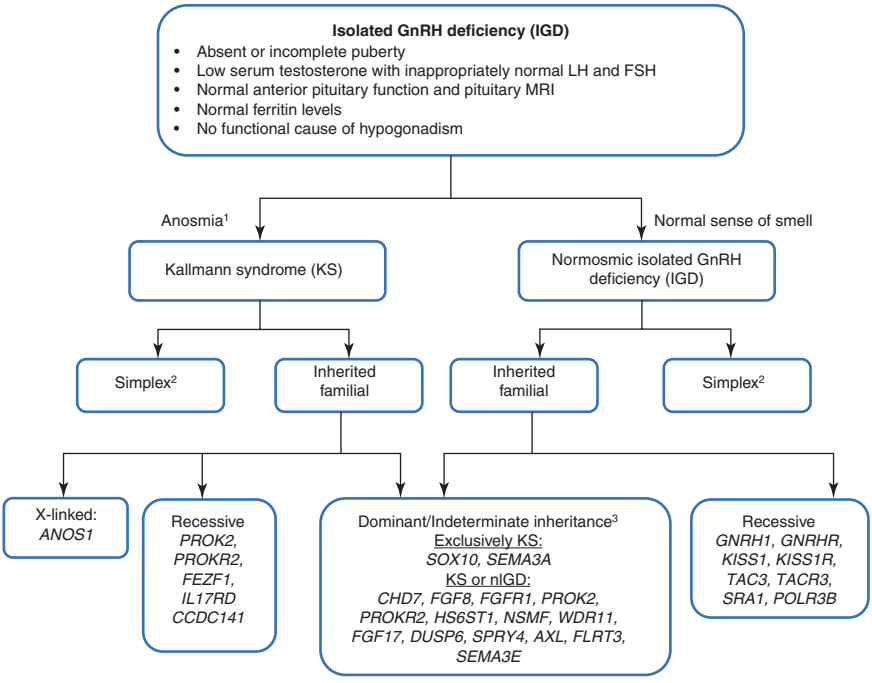
Replacement of deficient FSH can be achieved with use of recombinant FSH, though cost frequently limits utilization. Use of injectable human menopausal gonadotropin may provide sufficient FSH-like activity to promote spermatogenesis. It should be noted, however, that the efficacy of FSH replacement is not fully understood. As isolated FSH deficiency represents such a rare event, few studies have sufficiently addressed the role of hormonal replacement therapy as an effective treatment for infertility.

Genetic Defects of GNRH

Isolated GnRH Deficiency

Men with isolated gonadotropin-releasing hormone deficiency (IGD) will be found to have inappropriately low serum concentrations of both LH and FSH, with resultant low serum testosterone and impaired spermatogenesis. Absence of GnRH may be isolated (40%) or associated with anosmia (60%); when associated with anosmia, men are said to suffer from Kallmann syndrome. Kallmann syndrome will be discussed in the subsequent section. GnRH has been associated with a myriad of genetic defects (Fig. 19.2) [22]; as such, IGD may exhibit autosomal dominant, autosomal recessive, and X-linked modes of inheritance, with sporadic cases also being reported. Here we will discuss some better described genetic abnormalities. A general approach to the evaluation of men with a suspected IGD is depicted in Fig. 19.3.

Through these genetic abnormalities of developmental defects of GnRH neurons, impaired functional activity of these neurons, disorder of GnRH release, or



1. Microsmia or anosmia for age/gender using University of Pennsylvania Small Identification test (UPSIT)
2. Simplex refers to cases where apart from the proband. no other family member has a discernible GnRH deficiency and/or anosmia. A multi-gene panel or phenotype-specific approch will be appropriate for this group.
3. High likelihood of oligogenic inheritance. especially if incomplete penetrance of variants.

Fig. 19.2 Identified genetic defects contributing to the development of isolated gonadotropin-releasing hormone deficiency. (From Balasubramanian and Crowley [22], with permission)

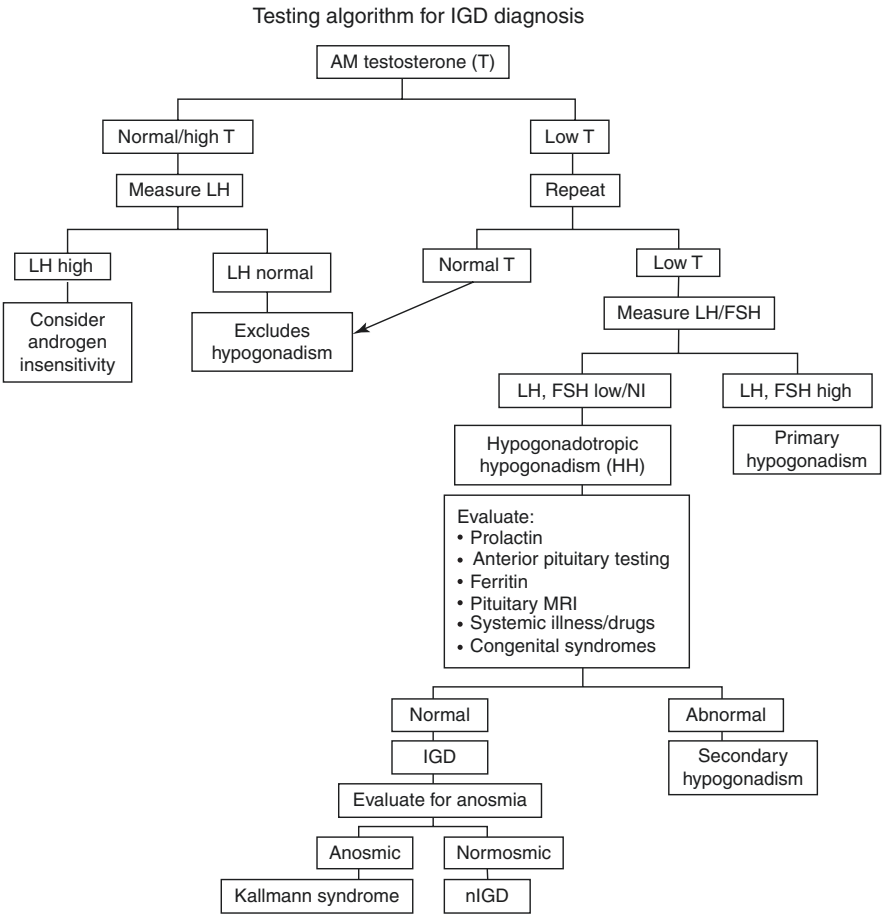


Fig. 19.3 Algorithmic approach to isolated gonadotropin-releasing hormone deficiency diagnosis. (From Balasubramanian and Crowley [22], with permission)

disrupted GnRH ligand and receptor interaction leads to isolated hypogonadotropic hypogonadism [23]. Inactivating mutations of the GnRH receptor represent the most frequent cause of normosmic IGD. Fibroblast growth factor receptor 1 (FGFR1) encodes a tyrosine kinase receptor, which interacts with anosmin-1 gene to participate in both GnRH neurite outgrowth and the development of the olfactory bulb, in addition to FGFR1 ligand, fibroblast growth factor 8 (FGF8). Inactivating mutations of either FGFR1 or FGF8 have been demonstrated in men with both Kallmann syndrome and normosmic IGD [23]. Also involved in olfactory bulb development and GnRH neuron migration are prokineticin receptor 2 (PROKR2) and its ligand, prokineticin 2 (PROK2). Numerous mutations to PROKR2-PROK2 have been reported, including heterozygous, compound heterozygous, and homozygous inactivating mutations [24–27]. Additionally, heterozygous mutations within

chromosome helicase DNA-binding protein 7 (CHD7) were identified in both men with Kallmann syndrome and normosmic IGD, accounting for approximately 5–10% of patients [28]. Errors in FGFR1-FGF8, PROKR2-PROK2, and CHD7 lead to an abnormal development of GnRH neurons, though this is not the only means in which GnRH activity may be affected.

Inactivating mutations in the G-protein-coupled receptor 54 (GPCR54) and its ligand, kisspeptin (KISSR), produced errors in GnRH secretion [29–31]. In the normal state, GPCR54 is a heptahelical transmembrane receptor expressed at the surface of GnRH neurons, which is then activated by KISSR to secrete GnRH. Additionally, homozygous frameshift mutations in GNRH1 gene, encoding the preprohormone of GnRH, also leads to normosmic IGD.

While most men with IGD are diagnosed in adolescence with absent or incomplete puberty, patients may present at any stage of development. In infancy, males with IGD may present with microphallus and/or cryptorchidism, reflecting the lack of androgens to direct sexual development. More often patients present at the time of puberty with abnormal sexual maturation, with a wide array of phenotypes observed. Physical exam usually reveals small, prepubertal testes, decreased lean muscle mass, and absence of secondary sex characteristics. Laboratory evaluation will reflect low serum testosterone with low gonadotropins. Most men will exhibit azoospermia on semen analysis, though in a variant of IGD pubertal development is preserved, and these men may have sperm in the ejaculate.

Treatment of men with IGD consists of exogenously replacing the lost gonadotropins. This is accomplished through the use of human chorionic gonadotropin (hCG) to replace LH and recombinant FSH to replace the lacking endogenous FSH. Alternatively, injectable human menopausal gonadotropin can provide both LH- and FSH-like activity.

In a 2014 study, Sidhoum and colleague showed that up to 10% of men with IGD may exhibit restoration of the hypothalamus-pituitary-gonadal axis after cessation of hormonal treatment; thereby, resumption of GnRH production and secretion are achieved [32].

Kallmann Syndrome

In 1856, Maestre de San Juan first documented an association between abnormalities in the olfactory bulb and microphallus [33]. However, it was not until 1944 that Kallmann and colleagues first reported on the heritance of this condition [34]. Thereafter referred to as Kallmann syndrome, the partial or complete absence of the olfactory bulb in hypogonadotropic hypogonadal men occurs in approximately one in 10,000 males. Kallmann syndrome has been noted to follow autosomal dominant, autosomal recessive, and X-linked modes of inheritance, with sporadic cases also being reported. Additionally, a significant amount of variability is seen across phenotypes. The association between hypogonadism and anosmia is one of development and proximity.

In early embryogenesis, GnRH neurons of neural crest and ectodermal origin are located within the olfactory placode. These neurons then migrate in close association with the axons of the olfactory receptor neurons to the hypothalamus. In a 1989 study, Schwanzel-Fukuda and Pfaff examined a 19-week aborted human fetus with known X-linked Kallmann syndrome [35]. The GnRH neurons were found arrested above the cribriform plate. They proposed that axonal elongation, path finding, and terminal differentiation were disrupted, indicating that a defect in cell targeting, innervation, and synaptogenesis was also responsible for the malformation of the olfactory bulb, thereby heralding the pathognomonic combination of hypogonadotropic infertility and anosmia.

Treatment of men with Kallmann syndrome consists of exogenously replacing the lost gonadotropins. This is accomplished through the use of human chorionic gonadotropin (hCG) to replace LH, in combination with recombinant FSH. Alternatively, injectable human menopausal gonadotropin can provide both LH- and FSH-like activity.

Mutations of the Androgen Receptor

The gene regulating the androgen receptor (AR) is located on the long arm of the X chromosome, within the banding region of Xq11–12 [16]. Male reproductive dysfunction has been associated with longer cytosine-adenine-guanine (CAG) repeat exon sequences. Men with AR insensitivity exhibit elevated serum testosterone, estradiol, and LH levels due to a loss of feedback inhibition at the level of the hypothalamus and pituitary. As FSH is under the control of inhibin feedback, serum levels are typically normal. It is thought that alteration in the testicular microenvironment is to blame for deficient spermatogenesis in the presence of normal gonadotropic stimulation of Sertoli cells. Tordjman and colleagues (2014) showed that paternity is possible via in vitro fertilization with intracytoplasmic sperm injection (IVF/ICSI) using sperm found within the ejaculate or retrieved by testicular extraction [36].

Hyperprolactinemia

Excessive production and secretion of prolactin disrupts the normal pulsatile release of GnRH from the pituitary gland, leading to downstream gonadotropin dysfunction and infertility. Men with multiple endocrine neoplasia (MEN) type I exhibit a predisposition to anterior pituitary tumors and thereby an increased risk of hyperprolactinemia. MEN type I is due to mutations located at chromosome 11q13, which encodes the 610 amino-acid protein, menin. Menin itself is involved with cell division, transcription regulation, and genomic stability. Along with anterior pituitary tumors, men afflicted with MEN type 1 also exhibit parathyroid and pancreatic islet

tumors. Over 1330 mutations (1133 germline and 203 somatic) of the MEN1 gene have been identified, with 23% consisting of nonsense mutations, 41% frameshift deletions or insertions, 6% in-frame deletions or insertions, 9% splice-site mutations, 20% missense mutations, and 1% whole or particular gene deletions [37].

While MEN type 1 represents a small portion of men with a genetic cause of infertility, given the propensity to develop other manifestations of the syndrome, it is imperative that the clinician consider it in a man with a personal or family history of endocrine tumors and a finding of hyperprolactinemia.

Review Criteria

- PubMed search included endocrine genetic defects, infertility, spermatogenesis ($n = 131$)
  - Articles were screened through title and abstract.

    Excluded articles related to nonhuman studies ($n = 72$)
    Excluded articles related to pertinent content ($n = 30$)
  - There were 29 articles cited from the initial search
- Secondary PubMed search for specific series regarding identified genetic disorders included Prader-Willi syndrome, Kallmann syndrome, and Klinefelter syndrome
  - Articles were screened through title and abstract

    Excluded articles that were redundant from initial search
  - There were additional four articles included
- A total of 33 total articles were cited

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Chapter 20
Sperm Aneuploidy

Lorena Rodrigo Vivó

Key Points

- Fluorescently labeled DNA probes are used to easily determine chromosome number in decondensed sperm nuclei. After sperm fixation and hybridization, fluorescence signals are evaluated and the spermatozoa are classified as haploid-normal, disomic, or diploid for the chromosomes included in the analysis.
- Males at risk of sperm aneuploidy are infertile *males with abnormal karyotype* (carriers of numerical abnormalities for sex chromosomes and carriers of structural rearrangements) and infertile *males with normal karyotype* (with impaired meiosis, with impaired sperm parameters mainly with severe oligozoospermia and nonobstructive azoospermia, with chemotherapy/radiotherapy treatments, with clinical history of recurrent implantation failures or unknown recurrent miscarriages, and with previous pregnancy with chromosomopathy).
- Sperm aneuploidy affects infertile males at clinical level (lower implantation and pregnancy rates and higher miscarriage rate after IVF/ICSI), at embryo level (high incidence of abnormal embryos and mosaicism), and at offspring level (higher risk of children with chromosomopathies for the chromosomes affected in the sperm).
- FISH in sperm allows the evaluation of infertility problems and genetic risk for offspring. When FISH is abnormal, genetic counseling should be offered, including the different clinical options available for the couple, such as prenatal testing, preimplantation genetic testing for aneuploidies, or sperm donation.

L. R. Vivó (✉)
PGD Molecular Cytogenetics, Igenomix, Valencia, Spain
e-mail: lorena.rodrigo@igenomix.com

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Case

Couple was with one abortion of the first trimester (at 8 weeks of gestation) in the last year. Pregnancy was through natural conception. Gestational sac was detected by vaginal ultrasound. Molecular analysis of products of conception showed an abnormal 45,X karyotype.

Familiar history: the female has one sister; the male has two brothers. There is no clinical history of abortions in the families of the couple.

Female history: she is 32 years old and with a normal 46,XX karyotype. She does not suffer from diseases. She has undergone one curettage in the last year under anesthesia. She works as a nurse. Menarquia is 12 years, menstrual formula: 5/28. Result of physical examination is normal, without congenital or acquired uterine anomalies. Results of trombophilic study (Factor II, Factor V, MTHFR) and immunological and endocrine tests are also normal.

Male history: he is 33 years old and with normal 46,XY karyotype. He works as a truck driver. Physical examination result is normal. He suffers from moderate oligozoospermia (sperm concentration: 10×10^6 sperm/mL). There is absence of Y microdeletions.

Most probable diagnosis is male factor infertility.

The abortion with 45,X karyotype and the moderate oligozoospermia suggested male factor infertility. FISH in sperm was done, and the analysis confirmed a high incidence of aneuploid sperm for sex chromosomes. Preimplantation genetic testing for aneuploidies (PGT-A) was recommended: nine oocytes were obtained, eight oocytes were correctly fertilized after ICSI, six blastocytes were analyzed by NGS, and two euploid blastocysts were obtained. One of the euploid blastocyst was vitrified. Single-embryo transfer of the other euploid blastocyst was done, leading to an evolutive pregnancy. Amniocentesis at 14 weeks of gestation showed normal 46,XY karyotype. The patient gave birth to a healthy child at 39 weeks of gestation.

Introduction

Infertility affects 10–15% of couples at reproductive age, with male factor causes responsible for 30% of these cases [1]. Intracytoplasmic sperm injection (ICSI) improves the chance of pregnancy in couples with severe male factor infertility. However, prenatal testing in ICSI pregnancies reveals a high incidence of de novo sex chromosome aneuploidies and structural rearrangements in couples with oligozoospermia [2, 3].

Sporadic chromosomal abnormalities appear in about 60% of early pregnancy losses [4]. Molecular analysis of first trimester miscarriages shows a higher incidence of aneuploid miscarriages (62.7%) from infertile couples achieving pregnancy by assisted reproductive technology (ART) compared to couples with natural conception pregnancies (40.6%). This incidence is even higher in couples with

severe oligozoospermia of <5 million sperm count, with up to 75% aneuploid miscarriages [5].

Aneuploidies in the embryo can arise during mitosis or can have a meiotic origin. Male meiotic origin of embryo aneuploidies occurs when an aneuploid sperm fertilizes an euploid oocyte. Therefore, clinical sperm aneuploidy testing can be used as a tool to determine possible causes of male infertility.

How to Analyze Sperm Aneuploidy: Technical Aspects

Chromosome studies in sperm were first possible in 1970 with the use of differential staining of specific chromosome regions [6]. Total aneuploidy rate estimated with this technique was considered excessively high and attributed to low chromosomal specificity of the technique. In 1987, a new technique allowed the fusing of sperm with hamster oocytes without zona pellucida, providing information of full chromosome content of sperm [7]. However, this technique was complex, laborious, and limited to the analysis only of fertilization-capable spermatozoa. Development of in situ hybridization techniques using specific radioisotope-labeled DNA probes in the mid-1980s [8] and the use of non-radioactive isotopes in the 1990s eventually allowed the standardization of fluorescence in situ hybridization (FISH) techniques to more accurately analyze sperm numerical chromosome abnormalities [9].

FISH uses fluorescently-labeled DNA probes directed to specific DNA sequences in the interphase sperm nuclei. This technique allows rapid and relatively simple evaluation of many sperms and can be used to identify structural and numerical chromosome abnormalities under fluorescence microscopy in ejaculated, epididymal, and testicular samples.

Nuclear chromatin in the sperm is highly condensed due to disulfide bridges between protamines. Previous to hybridization, spermatozoa must be fixed to maintain their morphology and allow permeability to DNA probes, and they must be spread on glass slides. The nucleus is then decondensed with reducing agents, and double-stranded DNA is denatured to allow fluorescent DNA probes to access and co-hybridize to specific complementary sequences.

Different combinations of centromeric, locus-specific, and subtelomeric fluorescent DNA probes are used for the FISH analysis of sperm. For segregation studies in structural rearrangement carriers, such as translocations and inversions, combinations of these types of probes are designed for each specific rearrangement. For carriers of numerical sex chromosome abnormalities, such as Klinefelter and XYY syndromes, and normal karyotype infertile males, the most widely analyzed are chromosomes whose aneuploidies are compatible with life, such as chromosomes 13, 18, 21, X, and Y [10]. However, other chromosomes can be added to the analysis when considering chromosomes related to meiotic paternal origin.

Fluorescence signals are visualized using a fluorescence microscope with specific filters for each fluorochrome. Analysis of fluorescence signal patterns can be manual or automated using signal analysis systems that include manual revision of

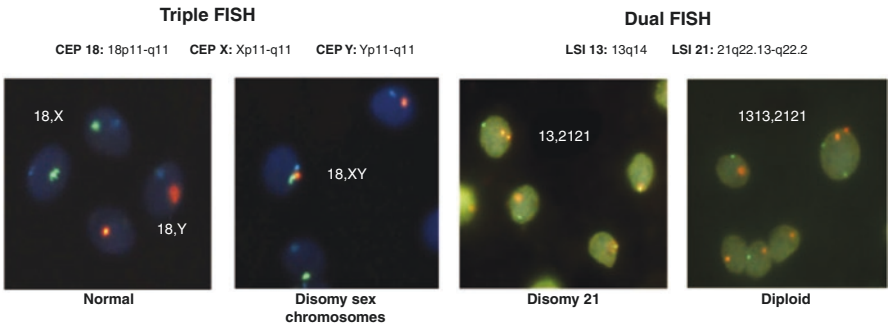

Fig. 20.1 Evaluation of FISH signals using epifluorescence microscopy. Spermatozoa are hybridized using a triple FISH with centromeric probes (CEP) for chromosomes 18, X, and Y and a dual FISH with locus-specific probes (LSI) for chromosomes 13 and 21. Spermatozoa with one signal for each of the autosomes evaluated and one signal for the sex chromosomes (X or Y) are considered as *normal haploid*, spermatozoa with two signals for one chromosome and one signal for the remaining ones are considered as *abnormal disomic*, and spermatozoa with two signals for all the chromosomes analyzed are considered as *abnormal diploid*

abnormal signal patterns. Using the criteria described by Blanco and coauthors [11], spermatozoa can be classified as follows (Fig. 20.1):

- Haploid-normal sperm: one signal for each evaluated autosome and one signal for sex chromosomes (X or Y)
- Disomic sperm: two signals for one evaluated chromosome and one signal for the remaining evaluated chromosomes
- Diploid sperm: two signals for each evaluated chromosome
- Nullisomic sperm: without signal for one evaluated chromosome and one signal for the remaining evaluated chromosomes

For clinical applications, it is recommended to score a minimum of 1000 sperms per chromosome analyzed, although this number may be smaller in ejaculated samples with low sperm count and in testicular samples from azoospermic patients. Nullisomes are often not considered in the analysis because they may represent a hybridization problem. An evaluated sperm sample is classified with an abnormal FISH result if it shows increased sperm with numerical chromosome abnormalities (aneuploidies and/or diploidies) when compared to a control population of normozoospermic fertile males.

Indications for Sperm Aneuploidy Testing

In a clinical scenario, FISH in sperm should be indicated in infertile males with higher risk of sperm aneuploidy. Regarding the karyotype, there are two groups where sperm aneuploidy testing may be indicated:

A. Infertile males with abnormal karyotype:

- *Carriers of numerical abnormalities for sex chromosomes*
  - *Klinefelter (47,XXY)* and *47,XYY syndromes* are at risk of low sperm production with poor sperm quality and abnormal chromosome constitution [12–15]. Blanco and coauthors [16] described incidences of 1–20% of spermatozoa with aneuploidies for sex chromosomes and 1% diploid sperm in these males.

- *Carriers of structural chromosome abnormalities*
  - Carriers of *balanced chromosomal rearrangements*, such as Robertsonian or reciprocal translocations and inversions, even with a variable range of alterations during gametogenesis, result in normozoospermia, oligozoospermia, or even azoospermia. After spermatogenesis, spermatozoa can be chromosomally unbalanced in a variable range [17]: 10–40% in Robertsonian translocations, 50–65% in reciprocal translocations, and 1–55% in inversion carriers [18].

B. Infertile males with normal karyotype:

- *Impaired meiosis in testicular analysis*
  - Infertile males with low recombination frequency in meiotic pachytene cells have a high incidence of aneuploid sperm [19, 20]. There is a significant correlation between cells with sexual vesicles without recombination sites and sex chromosome disomy in sperm [21]. Moreover, Peinado [22] described an abnormal FISH result in sperm in >80% of nonobstructive azoospermic males with low recombination levels, showing a 4-fold increase in disomy for all analyzed chromosomes compared to controls.

- *Impaired sperm parameters*
  - Classical meiotic studies of oligozoospermic males have revealed a direct correlation between abnormal chromosome pairing during meiosis and decreased sperm production [23, 24]. Moreover, several studies have revealed that the production of aneuploid and diploid sperm is associated with oligozoospermia [25–28], mainly at sperm counts of $<5 \times 10^6$ [29–31]. This correlation has also been observed in testicular sperm from azoospermic patients, mainly those with nonobstructive azoospermia [30, 32–37], where up to 42% of men have an abnormal FISH result [38].
  - This correlation is not as clear regarding sperm motility or morphology. Similar incidences of aneuploidy and diploidy are observed in spermatozoa with good motility compared to sperm with low motility [39] or nonmotile sperm [40]. However, others have described a negative correlation between sperm motility and increased sperm aneuploidy in small populations of males with severe asthenozoospermia with specific deformities involving sperm flagella [41, 42]. Regarding sperm morphology, some authors describe a 4-fold increase in aneuploid sperm in teratozoosper-

mic compared to normozoospermic males [43] and a 4.4-fold increase in aneuploid sperm in abnormal compared to normal morphologies [44], while others observe similar incidences of aneuploidies in sperm with different sizes and shapes in infertile males [45]. However, a general consensus seems to exist for a higher risk of sperm aneuploidy, diploidy, and polyploidy in severe teratozoospermia with large-headed and multiple-tailed spermatozoa [25, 46, 47].

- *Chemotherapy and radiotherapy treatments*
  - Depending on the type and duration, chemotherapy and radiotherapy treatments may have gonadal toxicity and may affect spermatogenesis to a variable degree. Therefore, 5-fold increases of diploid sperm and sperm with aneuploidies for autosomes and gonosomes have been observed after 6 months of treatment compared to basal levels [48–50]. In general, these rates decline to basal levels by 18–24 months post treatment [51]. Several studies have also described an association between Hodgkin's lymphoma and impaired spermatogenesis, some finding a significant increase in aneuploid sperm before any treatment [52, 53]. These data suggest that the emergence of cancer itself induces problems in meiosis.

- *Clinical history of unknown recurrent miscarriage*
  - Meiotic abnormalities [23, 24] and sperm aneuploidy have been reported in recurrent pregnancy loss (RPL) patients [29, 54–60]. Most reports describe an increased incidence of sperm with disomy for chromosome 18 and sex chromosomes and also incidence of hyperhaploidy [55]. Moreover, the proportion of men with increased aneuploid sperm is higher in couples with RPL [61].

- *Clinical history of repetitive implantation failure*
  - Several studies have related the presence of abnormal sperm FISH results with decreased pregnancy and implantation rates in ICSI cycles [62–64]. This was also observed in a study of patients with ≥3 failed ICSI cycles, where 31.6% of patients had an increase in spermatozoa with sex chromosome disomy [29].

- *Previous pregnancy with chromosomopathy*
  - Men with chromosomally abnormal offspring of paternal origin, such as Down syndrome (trisomy 21), Klinefelter syndrome (trisomy XXY), and Turner syndrome (monosomy X), have shown incidences of 1–20% aneuploid sperm for chromosomes affected in the offspring [65–68].

Impact of Sperm Aneuploidy

The first publications associating sperm aneuploidy with clinical outcomes described low ongoing pregnancy rates [32] or absence of pregnancy [69, 70] after conventional IVF/ICSI in infertile patients with high incidence of sperm aneuploidy.

Calogero and coauthors [71] described the absence of pregnancy after ICSI in 90% of infertile patients with an increased sperm aneuploidy rate. In couples with abnormal versus normal sperm FISH results, Rubio and coauthors [29] observed similar fertilization rates (74.5% vs 71.5%) but lower pregnancy rates (23.6% vs 36.5%) and higher miscarriage rates (80.0% vs 54.8%) after ICSI. Burrello and coauthors [63] showed similar results, with lower implantation rates in patients with >1.55% incidence of aneuploid sperm (13%) compared to patients with a lower incidence (34%).

Two additional small studies support an association between total sperm aneuploidy rate and clinical pregnancy. The first study by Petit and coauthors [62] shows a lower chance of clinical pregnancy in patients with higher total sperm aneuploidy rates, considered as total aneuploidy and diploidy rates—total sperm aneuploidy rate was 9% in couples without pregnancy after ≥4 ICSI cycles, 4.3% in couples with one pregnancy after 1–3 ICSI cycles, and 0.9% in fertile donors. Two years later, Nicopoullos and coauthors [64] described a 2.6-fold decreased probability of clinical pregnancy with every 1% increase in total sperm aneuploidy rate.

Preimplantation genetic testing for aneuploidies (PGT-A) in day 3 embryo biopsies and FISH analysis for a set of chromosomes shows a high incidence of chromosomally abnormal embryos (43–78%) and a high incidence of mosaicism (35–68%) in oligozoospermic and azoospermic patients with impaired meiosis or increased sperm aneuploidy [12, 72–78]. Sánchez-Castro and coauthors [77] described a correlation between the percentage of aneuploid sperm and embryos. Whereas the authors found 64.8% chromosomally abnormal embryos in patients with <20 million sperm count and 3% total sperm aneuploidy rate, they found a lower incidence of 41.1% aneuploid embryos in normozoospermic patients with 1.7% total sperm aneuploidy rate.

One year later, Rodrigo and coauthors [12] observed a different effect on embryos according to chromosome abnormalities in the spermatozoa. When sperm samples had an abnormal FISH result with an isolated increase of disomy for sex chromosomes, PGT-A showed increased embryos with aneuploidies for sex chromosomes but not an increase of triploid embryos. However, when sperm samples had an abnormal FISH result with an isolated increase of diploid sperm, PGT-A analysis showed a significant increase in triploid embryos but not an increase of sex chromosome aneuploidies. When sperm FISH analysis showed both increased sperm with sex chromosome disomy and diploid sperm, both aneuploidies for sex chromosomes and triploid embryos were increased. Therefore, clinical implications may vary according to the type of sperm abnormality. While spermatozoa with sex chromosome aneuploidies mainly result in aneuploid embryos compatible with life, diploid sperm results in triploid embryos that mostly miscarry before delivery.

Although most embryo chromosomal abnormalities miscarry or do not implant, several studies have reported ongoing pregnancies in which the fathers show increased sperm chromosomal abnormalities associated with chromosomopathies observed in their children. Blanco and coauthors [65] described high incidence of sperm with disomy for chromosome 21 (0.75% and 0.78%) in two men with children with Down syndrome, and paternal origin of the extra chromosome 21 was determined. Similar

reports in couples who miscarry or have children with aneuploidies for sex chromosomes, such as Turner syndrome or Klinefelter syndrome, have described high incidence of sperm with aneuploidies for sex chromosomes, ranging from 0.20% to 24.7% after sperm FISH analysis [66–68, 79–81].

Reproductive Counseling

FISH analysis of sperm as a clinical diagnostic tool for male infertility allows the evaluation of clinical consequences, such as infertility problems or genetic risk for offspring. When an abnormal FISH result is found, genetic counseling should be offered to the couple.

PGT-A has been proposed as an alternative to improve the possibility of healthy pregnancies in couples with abnormal sperm FISH results [72, 75, 76]. New approaches of embryo aneuploidy screening for all 24 chromosomes, such as array comparative genomic hybridization (aCGH) or next generation sequencing (NGS), offer improved clinical outcomes for different indications, including male factor infertility, compared with FISH analysis of a limited number of chromosomes [82]. Additional clinical options can be offered to the couple, such as prenatal testing in the case of slightly increased aneuploid or diploid sperm or sperm donation when severe meiosis impairment results in extremely high increases in abnormal sperm.

Review Criteria

- PubMed search for sperm aneuploidy in humans related to infertility and reproductive outcomes, chemotherapy and radiotherapy treatments, normal and abnormal karyotypes, and sperm parameters ($n = 1699$)
  - Articles were screened through title and abstract:

    Excluded articles related to animals
    Excluded articles related to carriers of structural rearrangements different from translocations, inversions, and gonosome aneuploidies
- A total of 82 articles were cited

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Part IV
Genetic Infertility: Is There Any Hope?

Chapter 21
Sperm DNA Fragmentation: Treatment Options and Evidence-Based Medicine

Ahmad Majzoub, Mohamed Arafa, Haitham Elbardisi, and Ashok Agarwal

Key Points

- SDF is a valuable tool for male fertility evaluation as it can influence fertilization rate and embryo development.
- The most commonly used SDF testing methods include terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), single-cell gel electrophoresis (the comet assay), the sperm chromatin dispersion (SCD) test, and the sperm chromatin structure assay (SCSA).
- Indications for SDF testing include unexplained infertility, recurrent miscarriage, varicocele, recurrent assisted reproductive failure and men with lifestyle risk factors.
- Lifestyle modifications, frequent ejaculation, antioxidants, varicocelectomy, sperm selection, and use of testicular sperm for intracytoplasmic sperm injection are among the treatments that can be performed in patients with high SDF levels.

Introduction

According to the World Health Organization (WHO), infertility in men or women is known as the inability to conceive after 1 year of unprotected intercourse [1]. About 15% of couples of reproductive age are affected by infertility, with male factors

A. Majzoub (✉) · M. Arafa · H. Elbardisi
Department of Urology, Hamad Medical Corporation, Doha, Qatar

A. Agarwal
Cleveland Clinic, Cleveland, OH, USA

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contributing up to 50% of the subfertility [2]. Male infertility is defined as a male's inability to produce a pregnancy in a fertile female. "Male factor" infertility is diagnosed when there is an alteration in sperm count, motility, and/or morphology in at least one of two semen samples, collected 1 and 4 weeks apart [1]. Known causes for male infertility account for 30–50% of the cases, while the remaining 50–70% have unknown causes and are termed "Idiopathic" [3]. It remains a struggle to diagnose and to treat idiopathic infertile men who eventually would require assisted reproductive techniques (ART) to overcome their infertility [4].

While semen analysis remains the cornerstone test for male fertility evaluation, it is not always an optimal predictor of the true male fertility status due to the intra-individual variation in sperm quantity and quality. Furthermore, up to 40% of infertile men have semen parameters falling within normal reference ranges [2].

A DNA fragmentation test is defined as the percentage of spermatozoa with fragmented DNA in the ejaculate [5]. Sperm DNA fragmentation (SDF) is currently recognized as an important cause of male factor infertility [3]. DNA is a vital component of the cell or spermatozoa, so high levels of SDF may affect various markers of conception, including embryo quality and blastocyst development [6–8].

The objective of this chapter is to describe the most commonly utilized SDF testing methods, highlight the clinical indications for SDF testing and to explore possible treatment methods for high SDF.

SDF Testing Methods

Several techniques are being used in the clinical setting to quantify DNA damage. The most commonly used methods include terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), single-cell gel electrophoresis (the comet assay), the sperm chromatin dispersion (SCD) test, and the sperm chromatin structure assay (SCSA). The first two methods can directly detect DNA fragmentation, whereas the latter methods analyze the susceptibility of chromatin to denaturation, giving an idea about the status of the nucleus in terms of integrity and compaction of chromatin. Therefore, each method can interpret a different aspect of sperm DNA activity.

TUNEL Method

The TUNEL method is considered one of the most promising tools for SDF testing [8].

This method can measure both single- and double-strand breaks using an enzyme that incorporates a modified and labeled dUTP at the 3'-OH terminal end of damaged DNA strands. The modified dUTP can be labeled by many ways: either directly with fluorescein or indirectly by using labeled antibodies or streptavidin. After collection and addition of the labeled dUTP, the sperm is examined under a microscope, and the amount of damaged DNA can be quantified [8].

The TUNEL method is considered to be the method of choice for the detection of damaged DNA caused by automated cell death (apoptosis). However, it is not specific to this detection as it can also detect cell death caused by other ways, such as exposure to chemicals or other toxins [9].

Single-Cell Gel Electrophoresis (The Comet Assay)

Similar to the TUNEL method, the comet assay can also detect single- and double-strand breaks in DNA (Fig. 21.1). It is a sensitive and rapid technique. It was first developed by Ostling and Johansson in 1984 and was later revised by Singh et al. in 1988 [10]. In this method, sperm cells are placed in an agarose gel plate so that all the proteins in the cells are lysed. The DNA is placed in an alkaline/neutral medium, and electrophoresis takes place. This allows the damaged and broken DNA fragments to migrate away from the nucleus at a faster rate than do the undamaged DNA fragments. After staining with fluorescent dye, comet shapes are shown, where the undamaged DNA fragments are referred to as the "head" of the comet and the migrating damaged DNA fragments are referred to as the "tail." The interpretation of this assay is as follows: the higher the number of tails is, the higher is the number of damaged DNA strands [10].

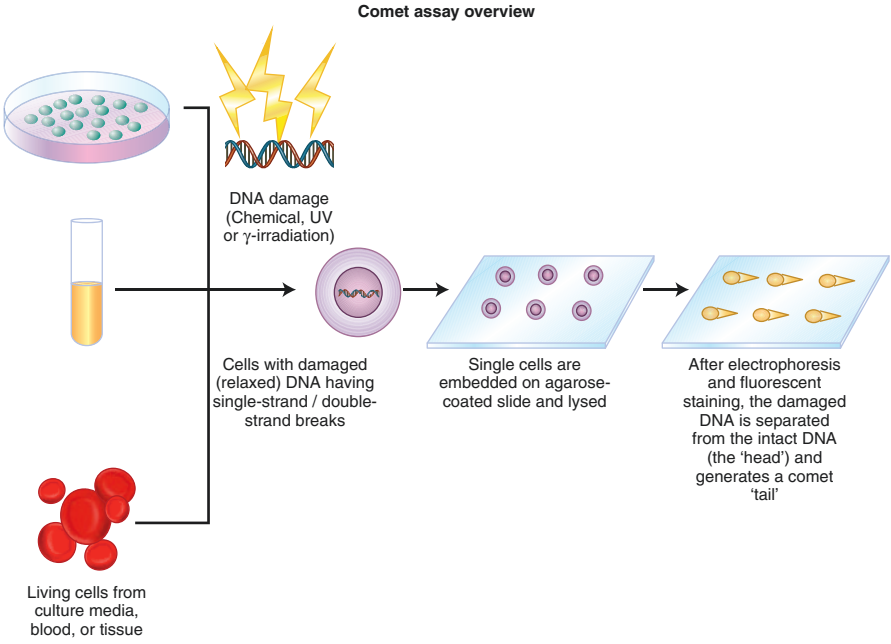

Fig. 21.1 Schematic illustration of the comet assay method

The Sperm Chromatin Dispersion Method (SCD)

The SCD method evaluates the ability of the sperm chromatin to disperse. It is a simple, fast, accurate, and highly reproducible method for the analysis of DNA fragmentation. Normally, upon the addition of hydrochloric acid, the sperm chromatin tends to denature and the acid will lead to the generation of single-stranded DNA. After this denaturation, a lysis solution is added, which will ensure the removal of all nuclear protein from the cell [11]. This procedure results in normal DNA spreading out of the center, producing halos that can be observed under the microscope [12]. However, fragmented DNA fails to show the same response to denaturation and lysis; hence, it does not form halos under the microscope. DNA fragmentation is therefore inversely proportional to the percentage of dispersion formed.

Unlike the TUNEL and the comet methods, the SCD method does not rely on the determination of color or intensity of fluorescence. Rather, it relies on the percentage of sperm cells with no dispersion (small or no formation of halos), which can be easily determined [13].

Sperm Chromatin Structure Assay (SCSA)

This method measures the susceptibility of DNA to denaturation, which occurs most commonly in fragmented DNA [7]. The SCSA is a flow cytometry test that measures two nuclear parameters simultaneously. After the addition of acid, fragmented DNA tends to denature to a higher extent than does normal DNA. Then an orange dye (acridine orange) is added to the solution, and flow cytometric analysis is performed. The sperm are passed under a beam of light with a specific wavelength, causing them to appear either orange (fragmented DNA) or green (normal DNA). A computer measures the percentage of green versus orange sperm cells, and a specific SCSA software plots the result, giving the two parameters: the DNA fragmentation index (DFI) and the percentage of sperm with high DNA stainability (HDS) [14].

A normal DFI is considered to be less than 15%. A sample with a percentage range of 16–29% is considered good or with fair fertility potential, whereas a sample with a percentage greater than 30% is considered to have a poor outcome for fertility [15].

As for the percentage HDS, it reflects the percentage of immature sperm, which is also predictive of pregnancy failure if elevated [15].

The advantages of using a flow cytometry test are high precision and accuracy, avoidance of human eye biases, and speed of measurement, in which about 250 cells per second can be assessed [14].

Indications of SDF Testing

SDF tests are increasingly being used in clinical practice for the evaluation of infertility in men. A recent survey of 65 professionals from 18 countries around the world reported that SDF testing is commonly ordered by 81.6% of responders who most commonly utilized the TUNEL and SCSA methods for SDF assessment [7]. This survey was part of a special issue on "Sperm DNA Fragmentation" in which we have identified specific clinical scenarios in which SDF testing would be most beneficial. These guidelines, endorsed by the society for Translational Medicine, have identified the following clinical indications for SDF testing:

Clinical Varicoceles

Varicocele is a vascular abnormality of the testicular venous drainage caused by the dilation and swelling of the pampiniform and/or cremasteric plexus. It is a very common condition prevalent in about 20% of the general male population [16]. While a good number of men with clinical varicocele are fertile, the condition is considered to be the most common correctable cause of infertility seen in about 40% of men with primary infertility and up to 80% of men with secondary infertility [16]. The impact of varicocele treatment on fertility status has been the subject of considerable debate. It is believed that proper patient selection is of utmost importance and that's where SDF testing may be beneficial. DNA damage from varicoceles can occur due to many factors mostly related to testicular hyperthermia and intratesticular blood stasis, resulting in hypoxia and oxidative damage [8].

Significantly worse SDF levels has been observed in infertile men with varicocele compared with counterparts without varicocele. This was associated with worse conventional semen parameters, early sperm apoptosis and abnormal mitochondrial membrane potential [17]. On the other hand, several studies have revealed a significant improvement in SDF levels after varicocelectomy that coincided with improved conventional semen parameters and most importantly with better pregnancy rates [18].

These findings lead Agarwal et al. to recommend SDF testing in patients with clinical varicocele to help in better selecting surgical candidates [8].

Unexplained Infertility, Recurrent Pregnancy Loss, or Intrauterine Insemination Failures

Male infertility may be present despite normal fertility evaluation and semen analysis. This occurs in 10–30% of couples seeking testing [8]. SDF testing in these men revealed a high DNA fragmentation index [8]. The same applies for couples with recurrent pregnancy loss and IUI failure [8].

SDF testing is indicated in men with unexplained infertility as studies have shown that even in men with normal conventional semen parameter results, high levels of sperm DNA fragmentation may be detected [8, 19, 20].

In a prospective study including 25 couples with unexplained infertility, the percentage of patients with SDF above 20% and 30% was 43% and 29%, respectively. All 25 couples were treated with ovarian stimulation and IUI. The proportion of couples who achieved pregnancy was significantly reduced when SDF rates were more than 20% [19].

Another study echoed similar results with successful pregnancy achieved at a higher rate (7–8.7 times) when the male partners had lower SDF levels [20, 21]. Saleh *et al*. observed that the SDF index, assessed by the SCSA assay, was higher in infertile men with normal SA (23%; interquartile range, 15–32%) than in fertile controls (15%; interquartile range, 11–20%) [22].

High level of SDF has been associated with recurrent spontaneous abortion, defined by two or more spontaneous miscarriages before 20 weeks of gestation. A study that evaluated 45 couples with RSA found that they have higher SDF rates (1.2 times) than controls (28.1 ± 4.9 vs. 21.7 ± 4.7, respectively; $P < 0.05$) [23].

Effect on IVF and ICSI

During conventional IVF, the prolonged exposure of the gametes to culture media would theoretically increase oxidative stress and the level of SDF, thereby imposing a risk on the IVF outcome. Conversely, during ICSI, the sperm is directly injected into the ovum, which utilizes its energy to repair any DNA damage right after fertilization [24]. This belief was, to a certain degree, proven by a number of systematic reviews reporting a significant negative impact for SDF levels on pregnancy rates with conventional IVF but not with ICSI [25, 26]. On the other hand, a significant relationship between SDF levels and miscarriage rate following both conventional IVF and ICSI has been reported [27]. A systematic review by Zini and Sigman showed that SDF was associated with a significant increase in the rate of miscarriage after IVF and ICSI with a combined OR of 2.48 (95% CI, 1.52–4.04; $P < 0.0001$) [28].

Risk Factors

SDF testing is indicated in men exposed to risk factors that can contribute to oxidative stress. Risk factors can be nonmodifiable, such as aging. Advancing age is associated with increased frequency of sperm DNA damage [6].

SDF testing can encourage infertile men to implant lifestyle modification to limit their exposure to modifiable risk factors, which include smoking, obesity, occupational exposure (lead and cadmium), organochlorine pollutants or pesticides (polychlorinated biphenyls and metabolites of dichlorodiphenyltrichloroethane), bisphenol A (compound widely used in plastic containers) [8].

A study evaluated the impact of cigarette smoking and alcohol consumption on semen parameters and sperm fragmentation measured by Halosperm. All parameters, including semen volume, percent of degenerated spermatozoa, and SDF, were significantly correlated with smoking status, and both smoking and alcohol consumption (separately or combined) were found to have deleterious effects on sperm parameters and SDF [29].

A study also correlated obesity with sperm DNA damage and found that the rate of sperm DNA damage measured by the TUNEL assay is increased in obese men with an odds ratio (CI of 95%) of 2.5 (1.2–5.1) [30].

Treatment

Conservative and Counseling Methods

Several conservative maneuvers can be performed aiming at reducing the SDF level. Ejaculatory abstinence time is believed to influence the SDF levels with shorter abstinence times through repetitive ejaculations, which have been found to lower SDF values [31, 32]. Agarwal et al. reported that a one- to two-day ejaculatory abstinence time resulted in significant reductions in SDF compared to longer abstinence. Although the ideal ejaculatory abstinence time is not yet determined, patients can be counselled to undergo repetitive ejaculations during the period of ovulation to minimize the effect of SDF on the likelihood of conception.

Patients can also be counselled to avoid risk factors that have been implicated in producing SDF. These include the following:

1. Physical factors such as radiation and heat, cigarette smoke, and airborne pollutants
2. Chemical agents such as anticancer drugs and sexually transmitted infections
3. Biological factors such as increasing male age, elevated body mass index, and diabetes

Infections should be controlled because several studies have shown that male genital infection and inflammation can increase SDF by 8–35% [33]. Inflammation can lead to the production of oxidative stress, which is known to cause DNA modification and damage.

Medical Treatment: Antioxidants

As mentioned before, oxidative stress is an important cause of SDF.

Antioxidants that are available in the semen are composed of enzymes such as glutathione peroxidase, superoxide dismutase, and catalase, as well as nonenzymatic compounds such as vitamins A, E, C, and B complex; pantothenic acid; coenzyme Q10 and carnitine; and micronutrients such as zinc, selenium, and copper. They provide protection against reactive oxygen species through either quenching or neutralizing their effects and maintaining a balanced redox potential.

Spermatozoa are particularly vulnerable to the harmful effects of reactive oxygen species (Fig. 21.2). They affect their activity, damage DNA structure, and accelerate apoptosis. Therefore, the use of antioxidants as a medical treatment for infertile men specifically those with SDF would be effective.

Antioxidants are compounds that could be consumed in the diet or can be taken as an oral supplement. They are the most common treatment prescribed for infertility, regardless of the cause [34, 35].

A study has shown that treatment of men with a DNA fragmentation index >30% with a 30- to 90-day course of antioxidant was associated with a statistically significant decrease in the DFI [36].

Many studies have shown the importance of antioxidants in infertile men and specifically in patients with SDF.

The combination of the following antioxidants has shown to improve sperm quality in terms of basic seminal parameters and DNA damage: L-Carnitine, vitamin C, CoQ10, vitamin E, zinc, vitamin B9, selenium vitamin B12 [37].

On the other hand, glutathione is a master antioxidant as it reduces oxidative damage by neutralizing the harmful free radicals. It has a synergistic effect with selenium. An observational study has shown that the use of glutathione for 2 months leads to a significant improvement in sperm concentration and a significant decrease in oxidative DNA damage [35].

Treatment of infertile men with docosahexaenoic acid (DHA) showed a significant decrease in SDF levels (p value <0.001), but there was an insignificant effect on semen parameters [38].

In addition to the previously mentioned antioxidants, L-carnitine has a pivotal role in cellular energy production; it necessary for mitochondrial oxidation of long-

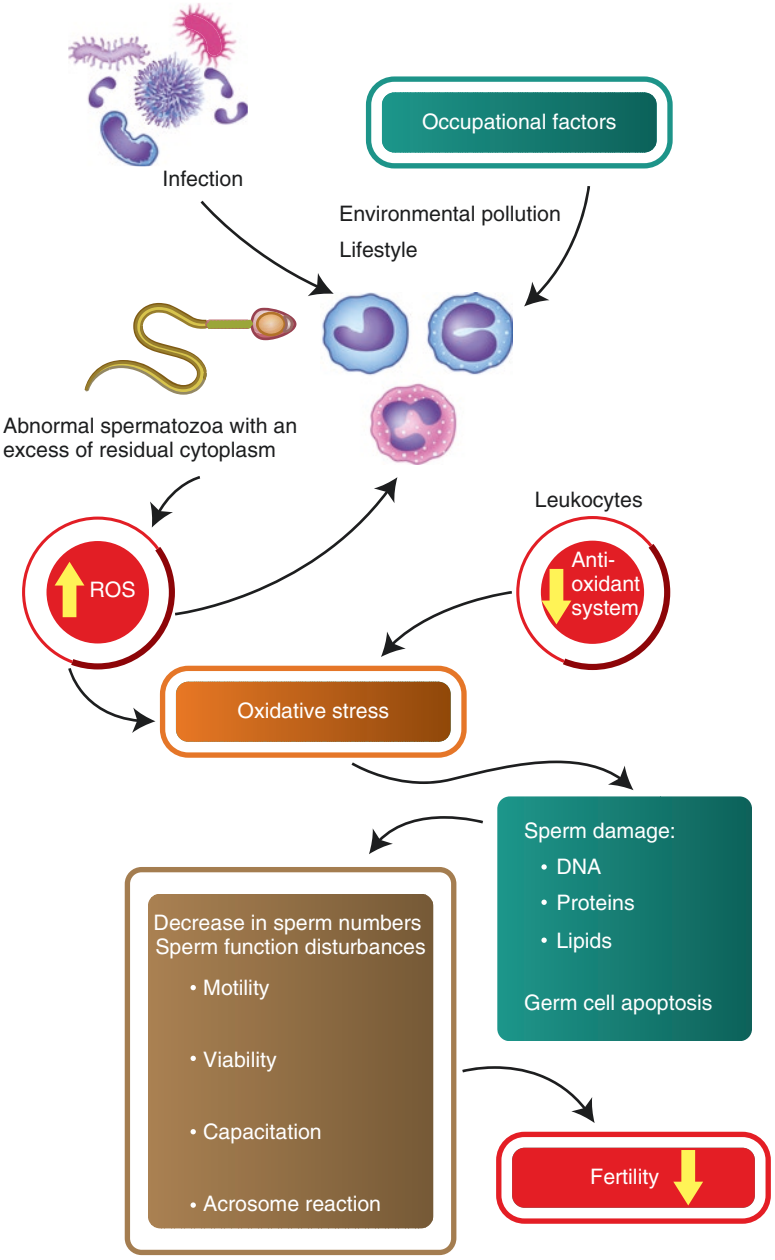

Fig. 21.2 The role of oxidative stress and antioxidants in male fertility

chain fatty acids. It also protects the cell membrane and DNA against damage induced by free oxygen radicals. The highest levels of L-carnitine in the human body are found in epididymal fluid, whose concentration is around 2000 times higher than in circulating blood [39]. A prospective observational study has shown that combining L-carnitine with vitamins C, E, B9, and B12; coenzyme Q10; zinc; and selenium results in decreased SDF levels, as well as increased sperm concentration in males with grade 1 varicocele [40].

Finally, lycopene, which is found in high levels in seminal fluid, provides protection against lipid and DNA oxidation and neutralizes ROS. A study published in 2015 was conducted on 21 normozoospermic males with idiopathic infertility and 23 males with semen abnormalities. After 3 months of therapy, the use of lycopene in infertile men has led to a significant improvement in the AA/DHA ratio in seminal plasma and has facilitated spontaneous pregnancy (16%), as well as IVF conception (42%) [41].

Surgical Treatment – Varicocele Ligation

Varicocele ligation, also known as varicocelectomy, is the most commonly performed surgery for the treatment of male infertility. This surgery can be performed at various anatomical levels, ranging from open to laparoscopic to microsurgical varicocelectomy [42]. The indications for varicocelectomy include the following: infertility with impaired semen parameters, hypogonadism, scrotal pain, testicular hypotrophy (mainly in children), or aesthetic issues with very large varicoceles [43].

Regarding varicoceles and infertility, the American Urological Association recommends that varicocele treatment should be given to the male partner of a couple attempting to conceive when all of the following are present: documented infertility, palpable varicocele, the female having normal fertility or potentially correctable infertility, and the male having one or more abnormal semen parameters or sperm function test results [44].

Varicoceles can be treated by either percutaneous occlusion/embolization (by the intravenous injection of specific materials to occlude the varicocele) or surgical ligation/clipping of the varicocele to prevent venous reflux [42].

Surgical ligation remains the most popular treatment of varicoceles, whereas percutaneous occlusion is reserved as a treatment option for persistent or recurrent varicoceles after surgical repair [42]. The effect of varicocelectomy on DNA damage was evaluated in an extended list of literature (Table 21.1).

A prospective study on 72 men with at least one-year history of infertility found that DNA fragmentation index (DFI) decreased significantly after varicocelectomy, from 34.5% to 28.2% ($P = 0.024$). All other sperm parameters (count, concentration, motility, and morphology) increased significantly [45].

Another meta-analysis on seven studies emphasized on the important role of varicocelectomy in restoring fertility, reducing DNA fragmentation, and concluded that it can improve sperm DNA integrity [46].

In a recent review on the role of varicocelectomy [47], Roque and Esteves concluded that the current evidence confirms the effectiveness of varicocelectomy as a means for both reducing oxidative stress, which results in sperm DNA damage, and potentially improving fertility [47].

Assisted Reproductive Treatment

Several treatments can be performed during the course of assisted reproduction in order to minimize or eliminate the detrimental effects of high SDF levels on the reproductive outcomes. These treatments include the following.

Table 21.1 Summary of studies evaluating the effect of varicocelectomy on sperm DNA fragmentation

Study	Design	Patients	Results
Zini, 2005	Retrospective cohort	37 patients with varicocele who had microsurgical subinguinal varicocelectomy performed	Mean SDF decreased after varicocelectomy (pre: 27.7%, post: 24.6%; $P = 0.04$).
Sakamoto, 2008	Retrospective cohort	30 infertile men with grade 2 or 3 varicocele (15 oligozoospermic and 15 normozoospermic) who had microsurgical subinguinal varicocelectomy performed	TUNEL-positive sperm decreased significantly 6 months after treatment (pre: 79.6%, post: 27.5%; $P < 0.001$).
Werthman, 2008	Retrospective cohort	11 patients with clinical varicocele and DFI >27% who had microsurgical subinguinal varicocelectomy performed	Ten of the 11 patients showed a significant decrease in SDF 3–6 months after varicocelectomy. Seven of the 11 patients showed a decrease in DFI to normal level, and the mean percent change in DFI was 24%.
Moskovtsev, 2009	Retrospective cohort	Patients with clinical varicocele treated with oral antioxidants alone (37 men) or subjected to both microsurgical subinguinal varicocelectomy and oral antioxidants (9 men)	SDF decreased in 78% of patients subjected to both varicocelectomy and oral antioxidants (pre: 44.7%, post: 28.4%; $P < 0.03$). No improvement in SDF was observed in patients on oral antioxidants alone (pre: 45.3%, post: 42.5%).

(continued)

Table 21.1 (continued)

Study	Design	Patients	Results
Smit, 2010	Prospective cohort	49 patients with clinical varicocele and oligozoospermia who had high inguinal ligation (36 men) or microsurgical varicocelectomy (8 men) performed	Improvement in SDF was observed after treatment (pre: 35.2%, post: 30.2%; $P = 0.019$). Thirty-seven percent of couples conceived naturally, and 24% achieved pregnancy with assisted reproduction after treatment. Mean postoperative DFI was significantly lower in couples who conceived naturally or with assisted reproduction than those who did not (spontaneous pregnancy: 30.1% vs 37.5%, assisted reproduction: 21.3% vs 36.9%).
Zini, 2011	Prospective cohort	25 patients with clinical varicocele and abnormal semen parameters who had microsurgical subinguinal varicocelectomy performed	Improvement in SDF was observed at 4 and 6 months after varicocelectomy (pre: 18%, 4 months: 10%, 6 months: 7%).
Lacerda, 2011	Prospective cohort	21 adolescents (ages 15–19) with grade 2 or 3 varicocele who had microsurgical subinguinal varicocelectomy performed	Sperm with intact nuclear DNA (comet class I) increased after varicocelectomy (49.6–64.5%, $P = 0.011$).
La Vignera, 2012	Not specified	30 patients with grade 3 left varicocele and oligoasthenoteratozoospermia who had microsurgical subinguinal varicocelectomy performed	There was significant reduction in SDF at 4 months after varicocelectomy (5.0–2.1%, $P < 0.05$), and postoperative results were similar to that of healthy controls (2.0%).
Li, 2012	Not specified	19 patients with clinical varicocele who had microsurgical subinguinal varicocelectomy performed	SDF was higher in men with varicocele than controls (28.4% vs 17.4%, $P = 0.007$). DFI decreased 3 months after operation (28.4–22.4%, P 0 0.018), and postoperative results were similar to that of controls.

Table 21.1 (continued)

Study	Design	Patients	Results
Baker, 2013	Retrospective cohort	24 patients with clinical varicocele who had microsurgical subinguinal varicocelectomy performed	SDF decreased after varicocelectomy (40.8–24.5%). A higher preoperative SDF was associated with a larger improvement postoperatively. Postoperative SDF in pregnant and nonpregnant couples showed no difference (22.2% vs 25.7%).
Kadioglu, 2014	Retrospective cohort	92 infertile patients with clinical left varicocele and abnormal semen analysis who had microsurgical subinguinal varicocelectomy performed	SDF decreased 6 months after varicocelectomy (42.6–20.5%, $P < 0.001$). A higher preoperative SDF was associated with a larger improvement postoperatively.
Ni, 2014	Prospective cohort	42 infertile men with clinical left varicocele and abnormal semen parameters who had microsurgical varicocelectomy performed	Higher DFI was observed in the preoperative group compared to controls (27.4% vs 11.5%, $P < 0.01$). DFI in patients who achieved pregnancy (20.6%) was lower than preoperative value (27.4%) and those of nonpregnant patients (24.7%). DFI in patients who achieved pregnancy after varicocelectomy was not significantly different from controls (20.6% vs 11.5%).
Pourmand, 2014	Randomized controlled trial	100 infertile patients with clinical left varicocele or subclinical varicocele who had varicocelectomy alone (group 1) or varicocelectomy, plus oral L-carnitine for 6 months (group 2)	Improvement in SDF was observed in both groups after varicocelectomy (group 1: 14.0–9.5%, group 2: 13.9–8.5%). The results were not different between groups.
Telli, 2015	Prospective cohort	72 infertile patients with clinical varicocele and oligozoospermia who had macroscopic inguinal varicocelectomy performed	SDF decreased after varicocelectomy (34.5–28.2%) with a mean follow-up of 6.2 months.

(continued)

Table 21.1 (continued)

Study	Design	Patients	Results
Tavalaee, 2015	Not specified	23 infertile patients with grade 2 or 3 left varicocele who had varicocelectomy performed	SDF improved 3 months after varicocelectomy (15.9–10.8%, $P < 0.001$).
Mohammed, 2015	Prospective cohort	75 infertile patients with clinical varicocele and altered semen parameters who had subinguinal varicocelectomy performed with loop magnification	Higher DFI was observed in preoperative patients than controls (32.4% vs 18.2%, $P = 0.003$). DFI deceased significantly after varicocelectomy (32.4–20.0%, $P = 0.05$). DFI in patients who achieved pregnancy at 1 year was significantly lower than that in patients who did not (16.4% vs 24.2%, $P = 0.04$).
Alhathal, 2016	Prospective cohort	29 infertile patients with clinical varicocele and abnormal semen parameters who had microsurgical subinguinal varicocelectomy performed	DFI was significantly higher in preoperative patients than controls (20.0% vs 7.4%, $P = 0.01$). DFI improved significantly after varicocelectomy (20.0–12.0%, $P = 0.001$).
Ni, 2016	Not specified	51 patients with clinical varicocele and abnormal semen analysis who had microsurgical retroperitoneal high ligation performed	SDF was higher in patients with clinical varicocele (range: 20.6–30.0%) compared to patients with subclinical varicocele (14.9%) and controls (12.0%). SDF reduced in patients with clinical varicocele and altered semen parameters, irrespective of clinical grade of varicocele. SDF was lower in patients who achieved pregnancy than in nonpregnant patients.
Abdelbaki, 2017	Prospective controlled cohort	60 infertile patients with clinical varicocele and abnormal semen parameters who had inguinal varicocelectomy performed with loop magnification	A higher DFI was observed in patients with varicocele than controls (29.9% vs 7.6%). DFI improved 3 months after varicocelectomy (29.9–18.8%, $P < 0.001$).

Sperm Selection Techniques

Sperm selection techniques are being recently employed in ART, most commonly in cycles of ICSI. These techniques are thought to improve the chance that structurally intact and mature sperm with high DNA integrity are selected for fertilization. These techniques include choosing the best spermatozoa according to surface charge, sperm apoptosis, sperm birefringence, sperm morphology under ultra-high magnification and ability to bind to hyaluronic acid [48]. Two techniques for excluding sperm with damaged DNA, namely, motile sperm organelle morphology examination (MSOME) and physiologic ICSI (PICSI) using hyaluronic acid-selected spermatozoa, received a significant amount of attention.

Studies investigating these sperm selection modalities have revealed conflicting results. Parmegiani et al. reported a SDF relative reduction by 67.9%, measured with SCD, while using PICSI [49]. While Rashki Ghaleno et al. reported that PICSI is an unreliable method for excluding sperm with high SDF prior to ICSI [50]. Similar findings were also reported in studies examining the effectiveness of MSOME [51, 52]. In a report evaluating 448 ICSI cycles from couples whose men were infertile due to high level of SDF, there were lower live-birth rates (24.2%) in the group with no intervention, compared to patients who underwent intracytoplasmic MSOME (28.7%), and PICSI (38.3%) [53]. The ability of other sperm selection techniques such as swim up technique and density gradient centrifugation to remove single and double strand DNA damage was tested. The results showed that such methods are equally efficient in eliminating spermatozoa containing double-strand DNA damage and sperm with highly damaged (degraded) DNA and that density gradient centrifugation is more efficient than swim up technique in selecting spermatozoa that are free from single-strand DNA damage [54].

Sperm Retrieval Techniques

The goal of sperm retrieval is to obtain sperm with best quality, adequate number for both immediate use and cryopreservation if possible, and to minimize the damage to the reproductive tract.

Sperm retrieval techniques are surgical methods originally developed to obtain spermatozoa from the epididymides and testicles of azoospermic men seeking ART.

However, their use in patients with high SDF stems from the understanding that in the majority of cases, such damage is accelerated during epididymal transit, indicating that the testicular sperm should contain lower levels of SDF than the ejaculated sperm. A few reports have confirmed this phenomenon by finding significantly higher levels of SDF in ejaculated sperm compared with testicular sperm [55, 56, 57].

Evidence shows that there is more DNA fragmentation in epididymal and ejaculated sperm than in testicular sperm [53]. In a systematic review and meta-anal-

ysis done in 2017 on five studies involving 143 patients, testicular and ejaculated sperm were compared for SDF. Clinical pregnancy rates were higher in the category of testicular sperm than in the category of ejaculated sperm, as were live-birth rates. On the other hand, miscarriage rates were lower with testicular sperm ICS [58].

We conducted a prospective study on 36 men with high-SDF levels who had a previous ICSI cycle from their ejaculates. A subsequent ICSI cycle was performed using spermatozoa retrieved through testicular sperm aspiration (TESA). Results of the prior ejaculate ICSI were compared with those of the TESA-ICSI. While there was no difference in the fertilization rate and embryo grading using ejaculate and testicular spermatozoa, clinical pregnancy was significantly higher in the TESA group compared to the ejaculated group (38.89% vs. 13.8%). Moreover, 17 live births were documented in the TESA group, and only three live births were documented in the ejaculate group ($p < 0.0001$).

The use of testicular sperm instead of ejaculated sperm assumes that the testicular sperm is of better quality. In comparing testicular to ejaculated sperm in the same patients, testicular sperm has been found to have lower SDF [59].

Conclusion

The role of SDF on male fertility has been a subject of great interest in this field of medicine. Several methods for SDF testing are available which is indicated in patients with clinical varicocele, unexplained infertility, recurrent miscarriage, assisted reproductive therapy failure and patients with lifestyle risk factors. Many interventions aiming to reduce SDF have been suggested including lifestyle changes, antioxidant use, varicocelectomy, sperm selection or use of testicular sperm prior to ICSI. Further studies are required to clarify the ideal treatment options for this group of patients.

Review Criteria
Extensive literature search was performed on search engines such as PubMed, Medline, Cochrane, Google Scholar, and ScienceDirect databases. Information from studies published for the past five decades until August 2018 was extracted. The literature search was limited only for the articles written in English language. “Sperm DNA damage and fragmentation” and “male infertility” were the main key terms used for conducting literature search. Book chapters and data published in scientific meetings relevant to sperm DNA damage were also included in this review.

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Chapter 22
The Potential of CRISPR/Cas Gene Editing to Correct Male Infertility

Douglas T. Carrell, Jingtao Guo, and Kenneth I. Aston

Key Points

- CRISPR/Cas gene editing tools allow for more efficient and precise manipulation of genomes.
- Further refinement of gene editing technologies is necessary to improve safety and efficiency before their adoption for routine use as a therapeutic.
- The accelerating discovery of mutations responsible for male infertility opens door for potential gene editing to restore fertility.
- Human spermatogonial stem cells (SSCs) are the only type of germline stem cells in human testis. A deeper understanding of SSC biology and a culture system to grow them in vitro will enable novel therapeutic options to restore fertility for infertile men.
- As gene editing technologies mature toward more routine clinical use, ethical considerations and careful oversight are critical for safe and responsible use of the technology for treating human disease.

D. T. Carrell (✉)
Departments of Surgery (Andrology) and Human Genetics, University of Utah School of Medicine, Salt Lake City, UT, USA
e-mail: douglas.carrell@hsc.utah.edu

J. Guo
Huntsman Cancer Institute and Andrology Lab, Department of Oncological Sciences and Surgery, University of Utah School of Medicine, Salt Lake City, UT, USA

K. I. Aston
Department of Surgery, University of Utah School of Medicine, Salt Lake City, UT, USA

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Introduction

Infertility is a complex disease that each year affects more than 24 million men globally [1]. While genetic causes are known for about 15% of male infertility cases, one of the most insidious characteristics of male infertility is that the majority of cases remain classified as idiopathic; hence, a great number of genetic pathologies remain to be identified [2]. Advancements in genetic technologies over the last two decades have been heralded as the means to unlock the causes of male infertility, potentially leading to new therapies to not only better treat male infertility but ultimately resolve the underlying genetic mutation with novel "gene therapy" techniques [3–6]. While that day has yet to arrive, progress is occurring, and at least in the realm of improvements in gene editing techniques, there has been a quantum shift that has made the objective more realistic in the near future.

Since the first understanding of the role of gene mutations in causing disease, gene therapy has been an ultimate goal in the treatment of many diseases and step-wise advancements have been made. In 1962, Szybalska reported the first case of transformation of a biochemical trait in a study in which he rescued cells by transferring functional DNA of the hypoxanthine-guanine phosphoribosyl transferase gene ($HGRPT^{+}$) into mutant ($HGRPT^{-}$) cells in a medium in which transformed cells could be selected [7]. Mario Capecchi, Oliver Smithies, and Martin Evans were awarded the 2007 Nobel Prize for their work on two fronts, which furthered progress in gene editing, homologous recombination, and the use of stem cells to evoke organism-wide genetic changes [8]. However, despite advancements in these and other aspects, gene editing remained a relatively low-efficiency process with a high risk of error. The recent introduction of novel "CRISPR/Cas" technologies has dramatically improved gene editing feasibility and reduced "off-target" errors and risks [9]. Additionally, CRISPR technologies are flexible and can be modified to expand into novel tools, including the use of epigenomic editing, to address novel genetic causes of disease, including infertility [10]. Early animal studies have already begun to address spermatogenesis defects and opportunities for advancements using CRISPR/Cas technologies [11, 12].

Human studies and therapies clearly require more assurance of safety and ethical and regulatory oversight; however, the use of gene editing to correct male infertility is beginning to come into focus. At least three strategies can be envisioned for the genetic therapy of male infertility: (1) in vivo gene therapy; (2) testicular biopsy with culture of SSCs, followed by in vitro gene editing and subsequent transfer back to the testis; or (3) in vitro gene editing with subsequent in vitro spermatogenesis and intracytoplasmic sperm injection (ICSI) into an oocyte (See Fig. 22.1). Currently, each of these pathways are evolving technologies with strengths and weaknesses, and it is impossible to predict the eventual therapies that will be most successful for the treatment of infertile males. This chapter reviews the building blocks that will be essential for the future implementation of gene editing technologies in a comprehensive and safe manner.

While there has been much hype about the wonders of CRISPR technologies, it is important to maintain an objective outlook on the pathway to gene editing for male infertility. In order for gene editing to become a real option in the treatment of male

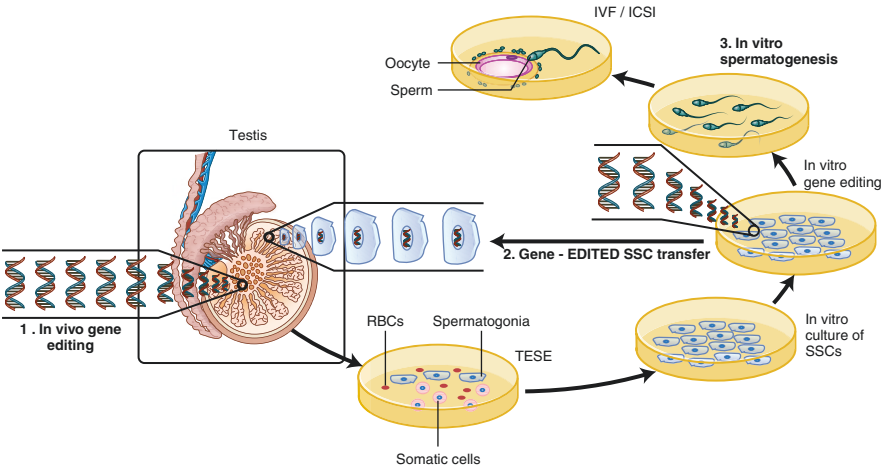

Fig. 22.1 Possible pathways to gene editing for male infertility. This figure demonstrates three possible pathways for implementing gene editing for male infertility. While in vivo gene editing using CRISPR/Cas technologies is the least invasive, in some respects, and most direct option, issues with the efficiency of targeting the SSCs and the effects of the blood–testis barrier present formidable hurdles to actual implementation. The most likely pathway involves isolation of spermatogonial stem cells (SSCs) from testicular biopsy tissue, with subsequent in vitro gene editing, followed by transplantation of the SSCs to the testes. Given the recent advances in induced pluripotent stem cell (IPSC) differentiation in vitro, it is conceivable that future efforts may include in vitro spermatogenesis of SSCs that have undergone in vitro spermatogenesis

infertility, advances need to occur in multiple areas. This brief overview will focus on three foundational areas for gene editing to succeed in treating male infertility. First, there is an urgent need for a better understanding of the genetic basis of infertility and the identification of specific variants that cause male infertility. Second, recent advances in our understanding of spermatogonial stem cell biology must be used to improve and refine in vitro SSC culture techniques. Lastly, there will certainly continue to be advances in CRISPR technologies that address improved safety and better efficiency, but ethical questions and strong oversight issues need to be resolved.

Gaining a Better Understanding of the Genetic Basis of Male Infertility

A critical requirement for the application of gene therapy strategies to the treatment of male infertility is clearly the identification of causal mutations. While significant strides have been made in this domain in the past few years, largely due to increasing accessibility of whole genome analysis approaches, the fact remains that an underlying genetic cause is undiscovered for a large majority of male infertility cases [3, 13, 14].

There are several major barriers to increased understanding of the genetic basis of male infertility. They include the molecular complexity of spermatogenesis, significant heterogeneity of the disease, challenges in classifying male infertility phenotypes, a lack of tools for functional validation of putative variants, limited sample resources, and limitations in funding relative to the magnitude of experiments that are required to make progress in this space.

The latter two challenges are being overcome with the formation of large consortia and collaborative efforts to study the disease, such as the NIH-funded Genetics of Male Infertility Initiative (GEMINI) (https://gemini.conradlab.org/) and the International Male Infertility Genomics Consortium (IMIGC) (http://www.imigc.org/ [15]), and an increased appreciation of the immense global cost of infertility treatments, which has motivated an increase in research funding by the National Institute of Health and other funding agencies.

Challenges in Studying the Genetic Basis of Male Infertility

Foundational to challenges associated with genetic research in male infertility is the inherent complexity of the processes required to produce functional sperm. Indeed, it has been demonstrated that the testis are among the most transcriptionally active tissues in the human body with nearly 16,000 human proteins (82% of all human proteins) expressed, including more than 2200 proteins that exhibit elevated expression in the testis (www.proteinatlas.org). Successful spermatogenesis requires the proper functioning not only of spermatogonial stem cells but also of other testicular cell types that form the proper niche for spermatogenesis, as well as appropriate endocrine signaling and response. Clearly there are many hundreds of genes whose disruption could result in an infertility phenotype.

With the concerted expression of several thousand genes being required to form normal and functional sperm, it stands to reason that the phenotype resulting from the disruption of any one gene will vary broadly depending on the pathways involved [16]. As expected, there are numerous and diverse male infertility phenotypes ranging from apparent normal spermatogenesis (based on tools currently available for assessment) to the complete absence of sperm in the ejaculate, termed azoospermia. Azoospermia can be the result of a physical obstruction or anatomic anomaly preventing the release of sperm (obstructive azoospermia (OA)) or the result of absence of sperm production in the testis (nonobstructive azoospermia (NOA)). Further, NOA can be characterized by the complete absence of germ cells in the testis (Sertoli-cell-only syndrome (SCOS)) or an arrest of spermatogenesis at any number of stages prior to spermiation (maturation arrest (MA)). In addition to azoospermia phenotypes, a reduced sperm count (oligozoospermia), reduced motility (asthenozoospermia) , increased abnormal sperm morphology (teratozoospermia), or any combination of these abnormalities is commonly observed in infertile men. Other abnormalities include microcephaly, macrocephaly, globozoospermia, defects in sperm

capacitation or acrosome reaction, defects affecting sperm binding, or penetration of the zona pellucida or fusion with the oolemma, among others.

The diverse and often uncharacterized defects impacting spermatogenesis and/or sperm function create significant challenges in properly phenotyping male infertility, further complicating the search for underlying genetic causes. The primary assessment of male fertility status is the standard semen analysis, which provides a rough quantitative measure of sperm production, viability, morphology, and motility [17]. The significant limitations of the semen analysis for diagnosing infertility are widely acknowledged [18, 19].

Ancillary tests aimed at characterizing the underlying defects associated with infertility include endocrine testing, sperm binding and penetration assays, tests of sperm DNA damage or seminal reactive oxygen species (ROS) load, sperm aneuploidy testing, and ultra-structural analysis [20]. These ancillary tools are used quite infrequently, and their diagnostic value is widely debated [21–23]. Clearly these tests can provide additional useful information when utilized appropriately, but there is no real consensus for the clinical indications for each of the tests with the exception of DNA fragmentation testing, for which clinical relevance is strongly supported by a growing body of experimental evidence [20, 21].

In addition to these tests, genetic screens, including Y chromosome microdeletion analysis and karyotype, are recommended in cases of severely reduced sperm production. These screens can successfully identify the underlying etiology in 15–25% of NOA or severe oligozoospermic men [5].

The primary mode of functional validation of variants presumed to play a role in male infertility is the generation of animal models, including drosophila, zebrafish, mouse, and others with the aim of recapitulating the mutation and associated phenotype. Knockout experiments in animal models have yielded a treasure trove of functional insights into the genes required for spermatogenesis and male fertility. For example, JAX lists nearly 400 mouse models that display a male infertility phenotype.

While animal models provide a valuable tool for the assessment of gene function in the context of male reproduction [24], there are significant shortcomings to a reliance on animal models. They include the high monetary costs and time associated with generating such models and the frequent inability to precisely recapitulate a genetic variant observed in humans because of species-specific sequence variation. The development of techniques to reliably culture human male germ cells in vitro will certainly open doors to expanded functional validation tools to accelerate our understanding of the genetic basis for male infertility.

The Search for Variants Responsible for Male Infertility

In spite of the diversity of, and limitations in, classifying male infertility phenotypes, significant progress in characterizing the underlying genetic basis of male infertility has been made over the past few decades, and progress is accelerating as genomic and bioinformatics tools improve. As mentioned previously, the role of YCMDs and karyo-

type abnormalities in severe spermatogenesis impairment (most notably Klinefelter's syndrome) has been known since the mid-1900s [25, 26]. Cumulatively, these genetic lesions account for 15–25% of men with NOA or severe oligozoospermia [27].

Since the discovery of those variants, targeted gene resequencing studies were the mainstay of male infertility genetic studies for many years, with extremely low yield. However, the past decade or more has witnessed extremely rapid advancement in tools that enable the interrogation of large swaths or even the entire human genome in a single experiment. This has enabled an unprecedented ability to search for genomic variants related to male infertility without a priori assumptions [28].

Early genome-wide experiments utilized single nucleotide polymorphism (SNP) microarrays or array comparative genomic hybridization (aCGH) to identify SNPs and copy number variants (CNVs) associated with a male infertility phenotype based on comparisons between infertile and control cohorts. These genome-wide association studies (GWAS) generally focused on NOA and severe oligozoospermia phenotypes as these represent the most severe forms of male infertility and are relatively easy to classify based on currently available diagnostic tools.

These studies were largely unsuccessful in identifying high-confidence variants; however, they were an important first step in characterizing the genetic architecture of severe impairment of spermatogenesis. Data generated through male infertility GWAS confirmed, as expected, that common variants do not contribute appreciably to male infertility disease risk, although the largest studies performed in Chinese populations identified SNP associations that reached genome-wide significance. However, two important trends were identified using array-based approaches. First, several groups recognized an increased genomic burden of large CNVs in infertile men compared with controls, and second, we observed increased regions of homozygosity in a subset of infertile men [29–32]. Both of these signatures suggest a genetic basis comprising rare and likely strong effect variants for male infertility.

The primary conclusion of array-based studies in male infertility was that approaches aimed at finding disease-causing variants would require the ability to identify rare variants or even variants unique to a single individual. With the precipitous drop in sequencing costs over the past decade, whole exome (WES) and whole genome sequencing (WGS) have become increasingly accessible for male infertility studies. In addition, large publically available human genome sequence databases such as ExAC (http://exac.broadinstitute.org/), with >60,000 exomes from unrelated individuals, serve as a powerful reference dataset for determining the background frequency of variants, thus significantly improving statistical power for identifying rare variants associated with male infertility or other diseases [33].

Variants Known to Cause Male Infertility

To date, the most productive strategy for identifying high-confidence variants associated with male infertility has been the application of WES or WGS to families in which two or more brothers share the same infertility phenotype, particularly in

families with elevated consanguinity. As larger, well-phenotyped male infertility cohorts are assembled, and analytical tools are refined, the power of cohort-based studies for identifying infertility-causing mutations will increase significantly. Table 22.1 displays the highest confidence, recurrent variants associated with various male infertility phenotypes.

Likely owing to the more discrete phenotypes, genetic variants responsible for qualitative sperm defects have been characterized in the recent years. Qualitative defects include globozoospermia, multiple morphological abnormalities of the sperm flagella (MMAF), including primary ciliary dyskinesia (PCD), acephalia, and macrozoospermia [3, 15, 34, 35].

Globozoospermia is a condition in which sperms lack acrosomes and are thus incapable of fertilizing and activating oocytes. Mutations in four genes, DPY19L2, PICK1, SPATA16, and ZPBP, have been identified in men with globozoospermia [3, 15, 34, 36–38]. Mutations in DPY19L2 (complete deletions of the gene in most cases) are identified in the majority of cases [37, 38].

Table 22.1 High-confidence genetic variants. This table highlights the genetic targets that have the highest confidence of being associated with male infertility, some of which are early targets for gene editing

Gene/region	Mutation	Phenotype	References
TEX11	Hemizygous FS, splice, and in-frame deletions	NOA	[46, 112, 113]
MCM8	Homozygous splice site mutation	NOA	[55]
SUN5	Homozygous or compound heterozygous point mutations, homozygous deletion-insertion	Acephalia	[43, 114, 115]
AURKC	Compound heterozygous deletion/point mutation, homozygous nonsense mutation	Macrozoospermia	[42, 116]
DPY19L2	Homozygous gene deletion or point mutation	Globozoospermia	[37, 117, 118]
SPATA16	Homozygous partial gene deletion, missense mutation	Globozoospermia	[36, 119]
DNAH1	Homozygous missense mutation, frameshift mutations	MMAF	[39, 120, 121]
AZF deletions	Microdeletion	NOA or oligozoospermia	[26]
Klinefelter syndrome	Chromosome gain	NOA or oligozoospermia	[122]
CFAP43	Compound heterozygous point mutations	MMAF	[41, 123]
CFAP44	Homozygous and compound heterozygous frameshift and point mutations	MMAF	[41, 123]
CATSPER1	Compound heterozygous insertion mutations	Asthenozoospermia	[124]
FANCM	Compound heterozygous frameshift/splicing variant, homozygous nonsense mutation	NOA	[57]

As the name implies, MMAF is characterized by sperm flagellar abnormalities and is accompanied by the absence of sperm motility. The best-characterized mutations associated with MMAF are in the gene DNAH1 [39]. Biallelic mutations in this gene result in one form of MMAF, PCD, in which the molecular structure of flagella and cilia is disrupted, resulting in defects in the sperm, as well as other conditions associated with ciliary defects such as chronic respiratory infections. Other genes previously implicated in MMAF include SEPT12, CFAP43, CFAP44, DNAH9, AKAP3, and AKAP4 [3, 13, 15, 40, 41].

Macrozoospermia is defined as large-headed and multiflagellated sperm, with all sperm displaying karyotypic abnormalities, most commonly tetraploidy. Mutations in AURKC are found in >80% of men with macrozoospermia [35, 42].

Acephalia is a condition in which sperm heads are detached from tails due to failure of formation of the centriole-tail attachment. Biallelic mutations in SUN5 appear to be responsible for approximately half of acephalic cases [43, 44]. A homozygous mutation in BRDT was also recently implicated in acephalia in a single patient [45].

The search for variants associated with quantitative sperm defects (NOA and severe oligozoospermia) has proven more complex due to the numerous genes that can impact sperm production efficiency. However, a growing list of NOA variants has been identified in recent years, with varying levels of evidence. These include TEX11 [46], TEX15 [47], SRA1 [48], MAGEB4 [49], DMRT1 [30], SPINK2 [50], NPAS2 [51], TDRD9 [52], TEX14 [53], MEIOB [53], DNAH6 [53], SYCE1 [54], MCM8 [55], CCDC155 [56], NANOS2 [56], SPO11 [56], WNK3 [56], and FANCM [57], and the list is growing rapidly.

Gene Therapy Candidate Genes

While the list of high-confidence variants causal of male infertility is certain to grow quickly through the efforts of male infertility consortia and the application of next-generation sequencing approaches, currently the number of mutations confidently implicated in male infertility is small. Given the current state of gene therapy approaches and our current knowledge of the genetic variants associated with male infertility, the number of potential targets is extremely limited, though this will certainly change in the not-too-distant future as our understanding of the genetic basis for male infertility increases and current gene therapy tools mature and new tools emerge.

Early gene therapy trials will likely target mutations responsible for NOA since there are currently no viable reproductive options for men with NOA who desire to use their own gametes. Additionally, currently available approaches are limited in their ability to modify large regions, so point mutations or small deletions are currently the most appropriate candidates. Mutations on X or Y or compound heterozygous mutations acting under a recessive model would be strongly favored as early candidates since the repair would only require modification of a single locus.

Ideally, recurrent mutations would be preferable as that would allow the application of a treatment to more than a single case. Lastly, targeting of genes that are only expressed in the testis would ameliorate the risk of unintended or unexpected consequences to some degree. Given these requirements, there is no ideal gene candidate for initial studies; however, suitable early candidate genes include TEX11, SUN5, and AURKC due to evidence of their role in male infertility, the nature of reported mutations, and their testis-specific expression.

Epigenetic Variation and Male Infertility

Sperm chromatin is uniquely packaged, compared to all other cells of the body. Briefly, about 95% of the genome is packaged with protamines, small basic proteins that silence DNA while packing the genome more tightly. The approximately 5% of the sperm genome retained associated with histones is limited to key developmental genes, some miRNAs, and imprinted genes. These developmental genes have a unique "bivalent" signature similar to some genes in stem cells, and it appears that these marks are associated with normal embryogenesis capability [58, 59].

Numerous studies have reported that the specific alterations of the sperm epigenome, including both aberrant DNA methylation signatures and abnormal histone modifications, are associated with male infertility [6, 60]. While these studies are associative and do not prove causality, it is possible that the loss of specific epigenetic marks on some key genes, causing gene silencing or overactivation, could be causal of male infertility. While further studies in this line of research are clearly needed, it is important to emphasize that such epigenetic variations may ultimately provide additional targets for the correction of male infertility.

Furthermore, it has been clearly shown that aging of the male is associated with increasing epigenetic alterations, including aberrations at genes associated with neuropsychiatric disorders such as autism, a genetic defect known to be associated with advanced paternal age [61, 62]. Additionally, specific environmental disorders are associated with some patterns of epigenetic alterations in sperm [63, 64]. Therefore, it is possible that future lines of gene editing may include the mitigation of epigenetic risk to offspring due to environmental factors. Such suppositions are very early at this point but of keen interest.

Understanding the Biology of Spermatogonial Stem Cells

Spermatogonial stem cells (SSCs) are the only type of stem cells in the mammalian male germline [65]. Unlike other types of stem cells, SSCs represent a unique mode in development: first, as stem cells, they need to balance between self-renewal and differentiation, which will give rise to their final functional product—mature sperm. Second, as part of the germline, they need to prepare themselves for the rapid and

fast transcriptional and epigenetic changes during early embryo development [66, 67]. Thus, SSCs not only serve as a great research model to study how stem cells develop but also provide knowledge to better understand and treat male fertility.

The study of SSCs in mice benefits greatly from the relatively easy and efficient transgenic system, which allows tissue- and cell-type-specific genetic perturbation, as well as lineage tracing [68, 69]. Moreover, the establishment of germ cell transplantation [70] and SSC in vitro culture system [71] offers even more opportunities to test hypothesis in vivo and in vitro synergistically. Thanks to those useful techniques, we now have a more complete understanding of how mouse SSCs develop physiologically and molecularly [65]. Based on histological studies, mouse SSCs are perceived as A_{single} (A_s) spermatogonia, which will amplify and proliferate to generate A_{pair} (A_p) and $A_{aligned}$ (A_{al}) spermatogonia [72]. These Type A spermatogonia then keep differentiation to become intermediate and Type B spermatogonia, which can enter meiosis upon activation by retinoic acid [65]. Both intrinsic and extrinsic factors are shown to play critical roles during mouse SSC self-renewal and differentiation. For example, GDNF, FGF, and CXCL12 are ligands secreted by niche cells to support SSC self-renewal, and ZBTB16, ETV5, and ID4 are important transcription factors, which are quite specifically expressed in mouse SSCs, and are involved in SSC self-renewal [65, 73–75].

In contrast, human SSCs are relatively less studied due to all the technical limitations and the lack of research materials. Although similar as they may firstly look, human and mouse SSCs are different in many aspects. Morphologically, in human testis, spermatogonia were perceived as A_{dark} (A_d) and A_{pale} (A_p), with A_d as the more quiescent stem cell population, which is different from the A_s, A_p, and A_{al} spermatogonia in mice [76–79]. Moreover, when researchers tried to culture human SSCs using the mouse protocol, these human SSCs lost their SSC and even germ cell identity within 3 weeks [80, 81]. Those observations indicate likely fundamental differences between the mechanisms underlying human and mouse SSC development. Due to the unsuccessful establishment of in vitro culture in humans, as well as the infeasibility of germline transplantation in humans, the study of human SSCs is even more lagged, which results in an urgent need to learn more from human SSCs. Although researchers already claimed the success in human SSC culture [82], the real identities of the cultured cells remained questionable, and there is lack of confirmation from other labs [65, 83]. As SSCs reside within the complex tubular architectures in the testis, one alternative way to grow SSCs in vitro could be to coculture them with other testicular cells, including the niche/supporting cells like Sertoli and Leydig cells, which can form self-assembled organoids that resemble testicular tubules [84, 85].

Recent advancement of high-throughput sequencing techniques [86], especially single-cell RNA sequencing (scRNA-seq), revolutionizes the way we investigate and perceive human SSCs and germline. Through profiling transcriptome at a single-cell level, scRNA-seq resolves several technical and conceptual challenges. First, traditional analysis of SSCs relies largely on enrichment by a cell surface marker via magnetic- or fluorescence-activated cell sorting (MACS or FACS) [87, 88]. Given that SSCs only comprise less than 1% of the testis, sorting out enough

SSCs for downstream analysis would require a relatively large amount of testis tissues to start with. However, with scRNA-seq, there is no need for cell enrichment, which saves large volume of tissues for repeat experiments or other purposes. Second, there are still debates that markers represent the most authentic SSCs [65]. Since scRNA-seq profiling does not rely on any kind of enrichment, it allows us to profile and analyze all the spermatogonia from testis and study their relationships with SSCs later. Third, scRNA-seq analysis offers a great opportunity to look for any cellular heterogeneity within human SSCs [89]. Last, through an unbiased single-cell transcriptome profiling from single cells from the whole testis, we can also see other types of germ cells and somatic cells within the human testis and their interactions with SSCs. Here, recent studies from several labs reported the single-cell transcriptomes from human SSCs and testis [90–92]. Those datasets and analysis provided researchers a roadmap to better understand human SSCs. For example, work from our lab showed that the human SSC is a heterogeneous population, and its development requires multiple cellular states transitioning from the most quiescent state to the highly proliferative and metabolic active state [87, 90]. Many signaling and transcription factors are involved in those critical transitions. Those discovered intrinsic and extrinsic pathways will serve as important guidelines for future human SSC culture.

In Vitro Culture of Spermatogonial Stem Cells

Using the knowledge drawn from the recent advancement in human SSC study, researchers are trying to culture human SSCs in vitro, which has both scientific and clinical significance. On the basic science side, if successful, SSC in vitro culture would provide unlimited research materials and a flexible platform for future studies and applications. This will greatly facilitate the current research of human SSCs.

On the clinical side, an in vitro culture system for human SSCs would be of great significance for germline stem cell therapy (GSCT) [93]. It is known that after the regimen, cancer survivors often suffer a significant higher risk of infertility [83]. Unlike adults, who can have offspring by preserving their sperm for in vitro fertilization (IVF) before the regimen, boys suffering cancer cannot do so since spermatogenesis does not commence then, indicating their loss of chance of having offspring forever. Thus, it is proposed as a means to help cancer survivors (especially childhood cancer survivors) to restore fertility via GSCT by taking small testicular biopsies from the cancer patient before the regimen, culturing their SSCs in vitro, and transplanting the SSCs back into the patient's testis after the regimen [93]. Those transplanted SSCs should be able to colonize and repopulate in the testis, which will undergo spermatogenesis, and there is no need to worry about immune response. Thus, the successful establishment of SSC in vitro culture is critical to the application of GSCT.

Of course, SSC culture is essential for some clinical applications of human SSC gene editing to treat infertility. Here, if the genetic causes of someone's infertility

are diagnosed, his SSCs can be cultured and undergo genetic manipulation to correct the mutated genes. (See Fig. 22.1.) After being transplanted back, those corrected SSCs should be able to generate normal and functional sperm. While in vivo editing remains an option, the advantages of in vitro editing include flexibility in applying multiple techniques and cycles, as well as safety to the patient.

Taken together, studying SSC biology has huge impact on both basic science and clinics. With the recent advancement, it is promising that the human SSC culture system will be successful in the near future.

CRISPR Technologies for SSC Gene Editing

Due to the benefits of the CRISPR/Cas system, gene editing technologies have recently advanced in accuracy, scope, and efficiency [94]. The early recombinant DNA technologies pioneered by Capechi and Smithies utilized relatively crude techniques that allowed for genetic manipulation via homologous recombination; however, the techniques were of very low efficiency (1 in 10–3 to 10–9 cells) and very inaccurate; genetic integration often occurred at nontarget sites [95]. While the success was improved by the introduction of the use of meganucleases to induce double-strand breaks, their range of flexibility in selecting target regions for strand breakage continued to be a problem, along with the issue of nonhomologous end joining (NHEJ), which has a high rate of inaccurate recombination [96, 97]. The introduction of zinc finger nucleases (ZFNs) and transcription activator-like effectors (TALEs) coupled with nucleases improved the flexibility and accuracy of target recognition but required cumbersome reengineering of the nucleases for each target site [98, 99]. Therefore, while the field advanced, the approach to clinical utilization was slow prior to the advances made in understanding the possibilities of CRISPR/Cas as a gene editing tool.

CRISPRs are clustered regularly interspersed short palindromic repeat DNA sequences that were first identified in *Escherichia coli* and are found in 40% of bacteria and 90% of archaea [100–102]. Within the bacterial genomes, CRISPRs are found adjacent to genes known as CRISPR-associated (Cas) genes, and the CRISPRs and Cas proteins together are used in bacterial adaptive immunity [103]. Beginning in 2007, the mechanisms of the CRISPR/Cas system began to be better illuminated, including the discovery that the Cas proteins are guided by specific CRISPR RNAs, leading the way to today's gene editing technologies that are much easier to use than the earlier nuclease systems, with improved accuracy and flexibility [94, 104]. Using a short guide RNA sequence, the Cas nuclease can be targeted accurately to very specific genome target sites (See Fig. 22.2 top).

While not included in this short review, several CRSPR/Cas systems exist in nature based on the Cas enzyme associated to the CRSPR and differ in many respects and in their potential usages [105]. These classes of Cas enzymes, as well as the use of engineered Cas molecules, are an ongoing focus of studies to facilitate better in vitro usage of the system via improved cellular integration, increased flex-

ibility of applications, and improved accuracy in the target sites. Since most CRSPR/Cas editing technologies rely on the use of viruses to integrate the CRSPR/Cas complex into cells, reduction of the large size of the CRSPR/Cas complex is a particular goal of engineering studies [106].

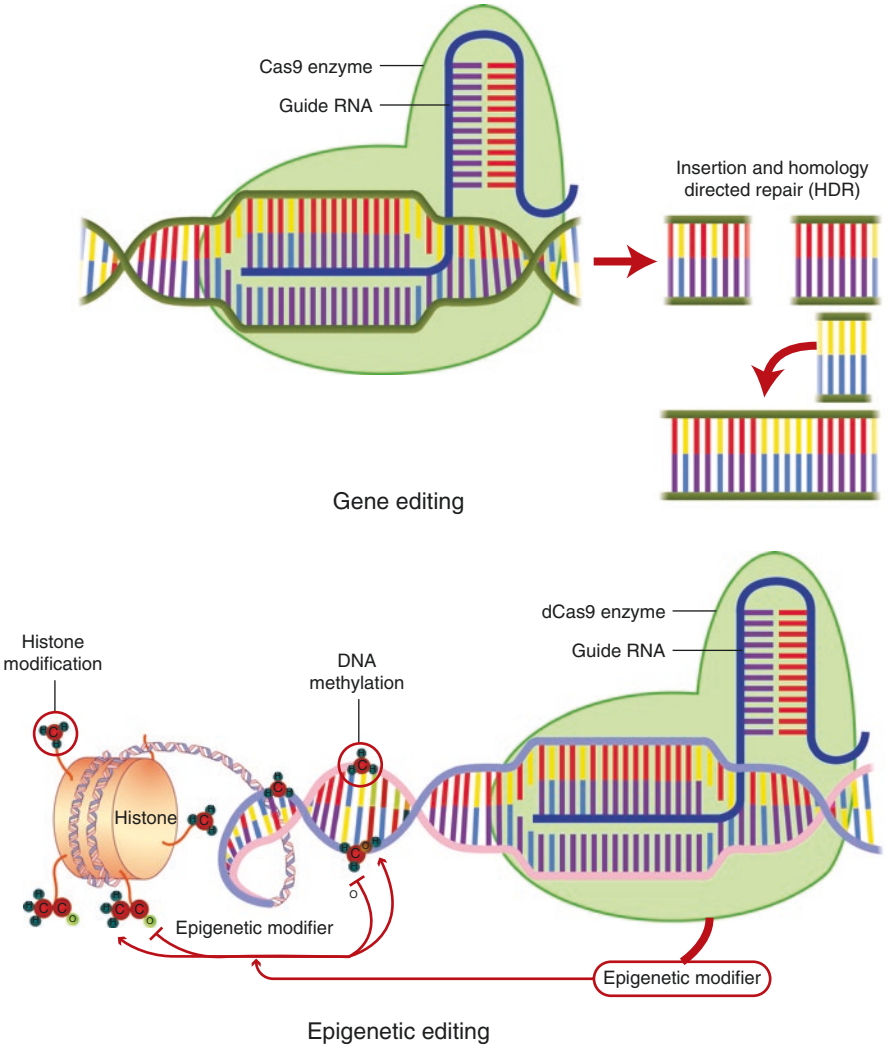

Fig. 22.2 Genetic and epigenetic editing using CRISPR/Cas. These diagrams illustrate in simplified fashion the basic mechanisms of CRISPR/Cas gene editing and epigenetic editing. In both cases, the Cas enzyme is directed to the target site by a guide RNA sequence. In genetic editing, the Cas 9 enzyme induces double-strand DNA breaks at the target site; however, in epigenetic editing, a modified Cas enzyme (dCas 9) is used that precludes double-strand breaks. Instead, the dCas 9 molecule is fused with an epigenetic modifier, such as TET or DMNT enzymes, which facilitate epigenetic modifications at the target site. In genetic editing, the target DNA is cut, then novel DNA can be inserted and undergo homology-directed repair (HDR)

The variability in the classes of Cas enzymes in nature, as well as the potential of novel Cas discoveries, facilitates a wide spectrum of potential usages of CRISPR/Cas technologies to areas beyond "traditional" gene editing. For example, one active area of research is epigenetic editing, in which specific epigenetic marks, be they DNA methylation or histone modifications, can be edited [94]. For example, the fusion of dCas, an inactive form of Cas, and DNA methyltransferase 3A (DNMT 3A) can cause site-specific DNA methylation [107]. Similarly, site-specific demethylation can be facilitated by the fusion of 10–11 translocation proteins (TET) and dCas with site-specific guide RNAs [108] (See Fig. 22.2 bottom). Given that epigenetic abnormalities are increasingly associated with male infertility, the application of CRISPR/Cas technology to address epigenetic defects is of interest and is feasible. Epigenetic signatures associated with male infertility may not be causal, but future CRISPR/Cas studies will help elucidate their role in infertility, as well as the possibility of correction. Furthermore, aging and environmental exposures are in some cases associated with specific abnormal sperm epigenetic signatures, thus opening the possibility of the correction of aberrant epigenetic signatures due to such exposures or lifestyle choices [63, 64]. While these applications are still in the conceptual stage, the potential for clinical implementation is apparent.

Given the advantages gained with the CRISPR/Cas systems of gene editing and the potential for curing serious disease, it is inevitable that the tools will be used in various applications in the future. However, reproductive germline editing offers particular advantages and opportunities. Given the current common use of testicular biopsy tissue to obtain sperm for ICSI, technologies already exist in the IVF lab for routine isolation of SSCs for in vitro technologies [109]. Furthermore, the transfer of SSCs back to the testis is a fairly routine process in animal models [110, 111]. The blood–testis barrier presents unique issues for in vivo gene editing and may preclude the ability for future in vivo gene editing success. However, future animal studies will address strategies to attempt in vivo technologies, as well as the more established in vitro techniques.

Conclusions

Recent publicity and hype regarding the future potential of CRISPR/Cas technologies to facilitate the advancement of gene editing prospects is well grounded in that they have facilitated significant improvements in ease of use, efficiency, accuracy, and flexibility. The prospects for its use to treat male infertility are clear; however, real hurdles remain, including the further identification of the key genetic variants that cause male infertility, since at present most cases of male infertility are not genetically defined. Emerging evidence suggests that those causes will include rare variants and complex multigenic causes, which will likely slow progress in identifying the causes then treating the patient through gene editing [3].

The flexibility of the CRISPR/Cas systems known in nature and the introduction of novel systems through engineering will likely lead to novel uses of CRISPR/Cas

beyond traditional gene editing. The most obvious is the potential for epigenetic editing, which is well into the investigation stage at present. Epigenetic editing will present novel challenges, such as maintaining stability, but offers the potential to address unique aspects of male fertility, including the effects of aging and environmental exposures to the father, which may convey risk to the offspring.

Furthermore, ethical and safety questions continue to exist regarding the usage of CRISPR/Cas technologies, including the risk of off-site changes, immunogenicity, and the scope of ethically acceptable usage and preuse approval processes. It is apparent that further advancements in safety and oversight issues are imperative. Nevertheless, it is clear that the advances already made recently have opened the door to the potential of real advances in the clinic, including in the treatment of male infertility.

Review Criteria

A thorough search of medical literature was performed using PubMed and Google Scholar. The keywords "genetics," "epigenetics," "exome," "GWAS," "CNV," "mutation," "CRISPR," "gene editing," "gene therapy," "male infertility," "spermatogenesis," "azoospermia," "NOA," "spermatogonial stem cells," "spermatogonia," and "single cell sequencing" were used for study identification and data extraction.

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